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Identification and Characterization of Novel Transporters Involved in the CO2 Concentrating Mechanism of Chlamydomonas reinhardtii

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IDENTIFICATION AND CHARACTERIZATION OF NOVEL TRANSPORTERS INVOLVED IN THE CO_2 CONCENTRATING MECHANISM OF *CHLAMYDOMONAS REINHARDTII*.

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LIST OF ABBREVIATIONS

Ambient/ Air	400 ppm (v/v) CO_2 in air
BST	Bestrophin
Ci	Inorganic carbon
CCM	Carbon dioxide concentrating mechanism
CA	Carbonic anhydrase
CCP	Chloroplast carrier protein
cDNA	Complimentary DNA
DIC	Dissolved Inorganic Carbon
EF3	Elongation factor 3
High CO_2	5% CO_2 in air (v/v)
LCI	Low CO_2 Inducible
Low CO_2	100 ppm CO_2 in air (v/v)
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
3-PGA	3- Phosphoglycerate
PS	Photosystem
pmol	Petamol
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RbcS	Rubisco small subunit
RbcL	Rubisco Large subunit
WT	Wild Type

ABSTRACT

The green alga *Chlamydomonas reinhardtii* possesses a CO_2 concentrating mechanism (CCM) which helps in successful acclimation to low CO_2 conditions. One of the main aspects of the CCM is bringing in inorganic carbon (Ci) into the cell as bicarbonate using Ci transporters. Current models of the CCM postulate that a series of ion transporters bring HCO_3^- from outside the cell to the thylakoid lumen where the carbonic anhydrase, CAH3, dehydrates accumulated HCO_3^- to CO_2, raising the CO_2 concentration for Rubisco. Previously, HCO_3^- transporters have been identified at both the plasma membrane (HLA3 and LCI1) and the chloroplast envelope (LCIA/NAR1.2), but the transporter thought to be on the thylakoid membrane has not been identified. The goal of this thesis has been to find the putative thylakoid transporter using a bioinformatics approach to identify candidate proteins followed by characterization of the interesting transporters using RNAi.

Nine candidate proteins were identified from the Chlamydomonas proteome which had transporter like domains and were upregulated in low CO_2. Three of the candidates are the paralogous genes (BST1, BST2, BST3) belonging to the bestrophin family, which are not only upregulated in low CO_2 conditions but their expression is controlled by CIA5, a transcription factor known to control Ci transporters of the CCM. YFP fusions demonstrate that all three proteins are located on the thylakoid membrane and interactome studies indicate that they might associate with other chloroplast CCM components. A single mutant defective in BST3, still grows normally on low CO_2, indicating that the three bestrophin-like proteins may have redundant functions. Therefore, an RNAi approach was adopted to reduce the expression of all three genes

at once. RNAi mutants with reduced expression of BST1-3, were unable to grow at low CO_2 concentrations, exhibited a reduced affinity for inorganic carbon compared to the wild type cells, and exhibited reduced inorganic carbon uptake. We propose that these bestrophin-like proteins are essential components of the CCM and deliver HCO_3^- accumulated in the chloroplast stroma to CAH3 inside the thylakoid lumen

Another mutant , A144 was discovered to be a part of a large-scale mutagenesis project to select for mutants that have been transformed with a paromomycin cassette and grow slowly under low CO_2 growth conditions. Using a modified form of the adapter PCR method, the location of the insert in A144 was found to be in a gene that codes for a protein with homology of fungal translational elongation factor (eEF3). This mutant cannot adapt to light after prolonged exposure to darkness and has a significantly lower rate of respiration than wild type. Thus we propose that EF3 putatively aids in the synthesis of stress proteins for dark adaptation.

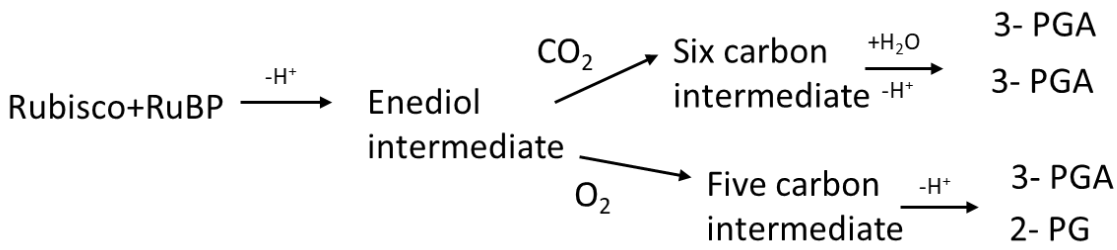
CHAPTER 1
LITERATURE REVIEW

Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase)

Photosynthesis is the process of converting solar energy to chemical energy using CO_2 and H_2O to form carbohydrates and oxygen in the chloroplast. ATP and NADPH, the products of light reaction are used by the “dark reaction” or the Calvin-Benson-Bassham (CBB) cycle of photosynthesis to fix CO_2 into carbon compounds. In the CBB cycle of photosynthesis, Rubisco fixes CO_2 to form two molecules of 3-phosphoglycerate (PGA)(see equation 1). This enzyme is present in all oxygenic photoautotrophic organisms and accounts for the gross productivity of 100Pmol of CO_2 fixation (Raven, 2009) and is one of the most abundant proteins on earth (Ellis, 1979) accounting for up to 50% of the soluble proteins in the leaves of C3 plants. In terrestrial plants, Rubisco is a very large enzyme made of eight large (RbcL) and eight small subunits (RbcS) with a molecular mass of 560kDa. The large subunit is encoded by the chloroplast genome and the small subunit is encoded by the nuclear genome. In plants and green algae RbcL have a molecular mass of 55kDa and RbcS has a molecular mass of 15kDa (Dean et al., 1989; Spreitzer, 1993). A hexadecameric (L8S8) form of the enzyme is found in plants, cyanobacteria, algae and many other eukaryotes (form I). Rubisco is a large and slow enzyme as it catalyzes only 3 reactions per second. The catalytic site of Rubisco is regulated by the reversible carbamylation of an essential lysine on the large subunit of the enzyme (Portis and Parry, 2007). This carbamate is stabilized by a Mg^{2+} ion as well as an interaction with non-carbamylated Rubisco with phosphorylated metabolites. Rubisco activase prevents this sort of inhibition and also

removes the inhibitor 2-carboxyarabinitol 1-phosphate (CA1P) from carbamylated Rubisco sites (Robinson and Portis, 1988; Portis and Parry, 2007).

The efficiency of CO_2 fixation by Rubisco is limited by an oxygenation reaction as shown in equation 1(Andersson and Backlund, 2008).



(1)

To overcome a low affinity for CO_2 coupled and the oxygenase activity, plants produce a large amount of the Rubisco protein. Rubisco evolved when primordial the earth's atmosphere was rich in CO_2 with very low levels of O_2. With the development of photosynthesis, levels of CO_2 dropped, and carbon was fixed and buried eventually becoming fossil fuels or $CaCO_3$ deposits. Evolution of photosystem II was another major development which produced O_2 and raised its levels to 210 mbar from nearly undetectable levels (Sage, 2004; Govindjee and Shevela, 2011; Moroney et al., 2013). The rate of carboxylation and oxygenation depends on the specificity factor of Rubisco. The specificity factor is defined as V_cK_o/V_oK_c such that V_c/V_0 are maximal velocities of each reaction and K_c/K_o are the Michaelis constant for each substrate (Laing et al., 1974). The turnover rates and specificity factors vary amongst Rubiscos of different organisms. For instance, Rubisco from some bacteria have high turnover rate and low specificity factors but plants and green algae have high specificity factor and low turnover rates (Jordan and Ogren, 1981; Bainbridge et al., 1995). Aside from the specificity factor, the concentration of each substrate also plays a role in turnover rate of

carboxylation along with temperature (Andersson and Backlund, 2008). Thus higher plants have a more specific but slower Rubisco than cyanobacteria (Jordan and Ogren, 1981).

The oxygenation reaction of RuBP forms one molecule of phosphoglycolate and one molecule of 3 -phosphoglycerate. Phosphoglycolate has to be reclaimed by photorespiration. Photorespiration reduces carbon loss from the oxygenation reaction of Rubisco. Nearly 75% of the carbon potentially lost by oxygenation is restored by this process as shown in fig.1.1. Briefly, phosphoglycolate phosphatase dephosphorylates phosphoglycolate to glycolate, which is moved from the chloroplast to the peroxisome (Bauwe et al., 2010; Moroney et al., 2013). In the peroxisome it forms glyoxylate via glycolate oxidase which ultimately forms glycine. The lost carbon is the CO_2 released when two glycines make a serine and ammonia. Despite being energy intensive, photorespiration is important for the plant. Evidence shows even organisms which accumulate inorganic carbon (Ci) in high concentration struggle to survive when the photorespiratory pathway is disrupted.

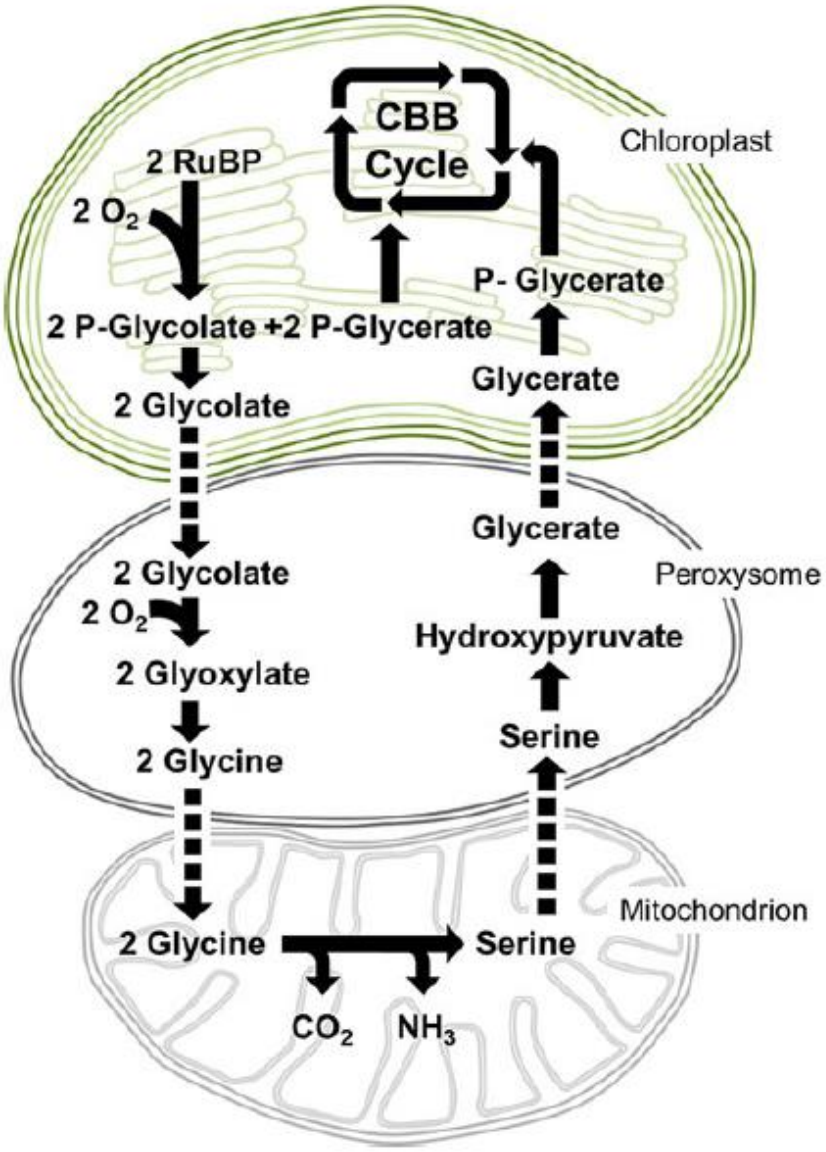


Figure 1.1. Outline of the photorespiration in plants (Moroney et al., 2013). The major carbon intermediates and compartments have been emphasized. CBB Cycle Calvin–Benson–Bassham cycle. Transport has been shown by dashed lines.

Why did Rubisco not evolve to reduce or minimize oxygenation? There are no direct explanations for this except for two probable hypothesis (Moroney et al., 2013). Two possible hypotheses worth reviewing would be to reason that a simple mutation cannot effectively change the enzyme to minimize or eliminate oxygenation because

there are no real binding sites for CO_2 and O_2, Rubisco essentially stabilizes the enediol intermediate formed from RuBP and allows either gas to react with this intermediate (Spreitzer, 1993; Cleland et al., 1998; Spreitzer and Salvucci, 2002). The six-carbon intermediate formed from the carboxylation reaction binds Rubisco more tightly and this is how Rubisco favors the CO_2 reaction. Since both gases are small uncharged molecules which diffuses across membranes easily,mutations minimizing diffusion by a would also be complicated and extremely unlikely (Moroney et al., 2013). In higher plants, which have a specific enzyme, the binding of the six carbon intermediate is relatively stable and tight which makes the enzyme slower than the enzyme in cyanobacteria. A second hypothesis is that the oxygenation reaction is regarded as an outlet for excess ATP and NADPH generated in the light reactions especially when the rate of carboxylation is low at high temperature or under drought conditions (Kozaki and Takeba, 1996). Lack of oxygenation during these times might cause over reduction of the electron transport by forming superoxide species that can damage the plant. Microalgae, diatoms and cyanobacteria have additional problems of CO_2 availability due to the slow rate of diffusion of CO_2 in water versus air. Thus, several photoautotrophic organisms such as marine algae, C4 plants and CAM plants have adopted methods to minimize oxygenation by increasing the CO_2 concentration around Rubisco. These adaptations include sequestering Rubisco in a compartment to reducing the leakage of concentrated CO_2. The following sections will go into details of such adaptations.

CCM (Carbon dioxide concentrating mechanisms)

There are two main ways by which photosynthesis can be made more efficient. One way is to reduce oxygen concentration at the site of Rubisco, which is difficult, due

to oxygen being constantly produced by PSII and the fact that atmospheric oxygen is so high. The other method is known as the carbon dioxide concentrating mechanism (CCM) which is an adaptation to increase the concentration of CO_2 in the vicinity of Rubisco. CCM's in marine algae like cyanobacteria, diatoms and Chlamydomonas accounts for 50% of carbon fixed per year in oceans. C4 and CAM require a process of carboxylation–decarboxylation to increase CO_2 concentration at Rubisco. For more information see (Keeley, 1998; Sage, 1999, 2004; Raven et al., 2008; Moroney et al., 2013; Winter and Holtum, 2014) and fig 1.3.

The main features of the CCM, as shown in fig.1.2, are as follows: - a) Compartmentalization of the Rubisco in a location where CO_2 can be concentrated to minimize oxygenation. b) Bringing in inorganic carbon in the form of charged molecules like HCO_3^- which does not diffuse across membranes like CO_2 c) Carbonic anhydrases that are strategically placed such that Ci forms can be interconverted to ensure CO_2 concentrates around Rubisco. d) sensing the CO_2 signal and expressing CCM genes in limiting CO_2.

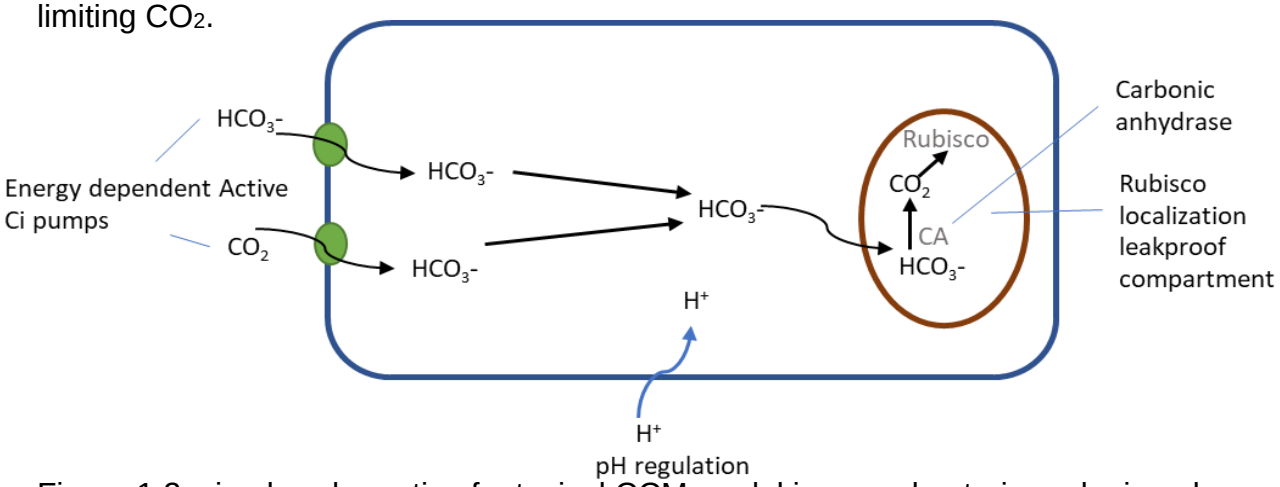


Figure 1.2. simple schematic of a typical CCM model in cyanobacteria and microalgae (Wang et al., 2015). Energy dependent bicarbonate transporters bring Ci (inorganic carbon) into the cell which is converted to CO_2 via carbonic anhydrase, which are strategically localized to minimize CO_2 leakage, inside a leakproof compartment where rubisco is localized.

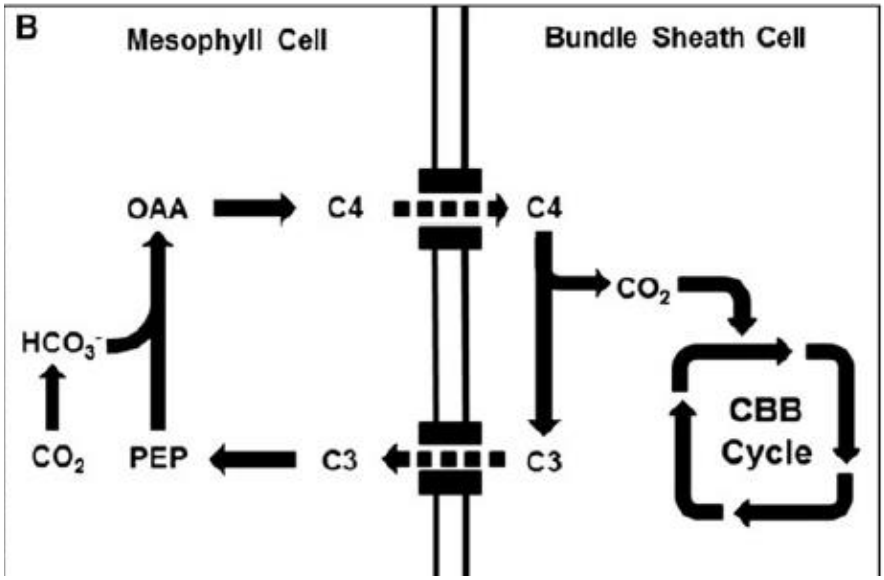


Figure 1.3. The C4 pathway of photosynthesis (Moroney et al., 2013). CBB- Clavin Benson Bassham cycle. OAA- Oxaloacetate, PEP- Phosphpphenolpyruvate, C3- puryvate, C4- Malate/ aspartate.

-**Cyanobacterial CCM**

The main features of the CCM in cyanobacteria are a Ci transport system, a microcompartment to compartmentalize the Rubisco and strategically placed carbonic anhydrases (CA) which convert the Ci forms at the site of Rubisco localization. Unlike C3 terrestrial plants cyanobacteria are saturated at ambient CO_2 levels. Cyanobacteria Rubisco has a low affinity for CO_2 with a K_m >150 µM and a high V_{max} (Badger and Andrews, 1987). Ambient CO_2 concentration in water is less than 15 µM, so cyanobacteria have an obligate need to have a functional CCM. Some of the main features of a cyanobacterial CCM are discussed in the following sections and a schematic diagram shown in fig.1.4 :-

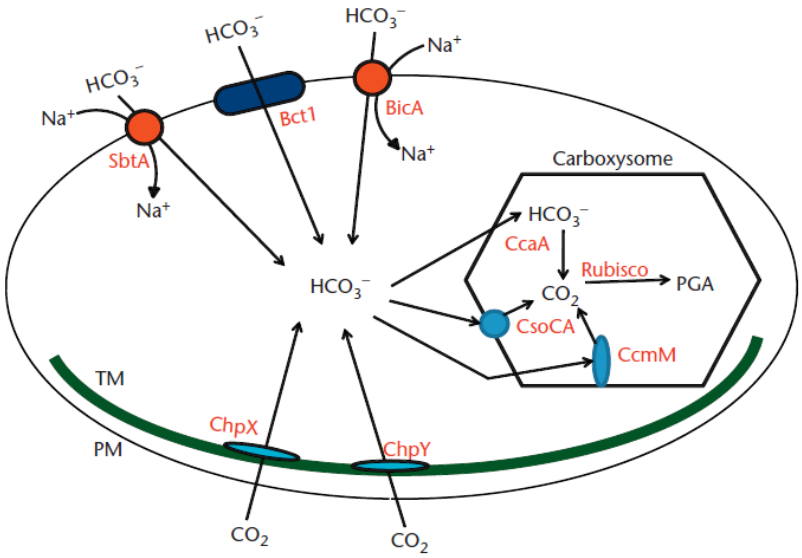


Figure 1.4. Schematic representation of the CCM in cyanobacteria (Mukherjee, 2013). Arrows indicate the direction of Ci flow. The hexagonal structure is the carboxysome where Rubisco is localized.

Ci transporters

There are five different Ci uptake systems in place in cyanobacteria which include three HCO_3^- transporters and two CO_2 uptake systems as shown in fig. 1.4. Ci uptake and accumulation requires energy and most of this energy is obtained from high energy molecules of the light reactions of photosynthesis. Some energy sources can be ATP (BCT1), NADPH/reduced ferredoxin for uptake of CO_2, or a sodium gradient (Price et al., 2004). Cyanobacteria can accumulate Ci internally 1000-fold higher compared to ambient Ci. Due to an absence of carbonic anhydrase in the cytosol Ci is accumulated in non-equilibrium with ambient CO_2 (Badger and Price, 1989). The cytoplasmic pH of cyanobacteria is 7.8-8.4 which makes it easier for HCO_3^- to accumulate. Being a charged molecule, it does not leave the cell as easily as CO_2 which is a small

uncharged molecule. There is considerable variation amongst the Ci flux and affinity of the various uptake systems. Some transporters are constitutive in expression and some are induced by low CO_2. β cyanobacteria which are freshwater and estuarine species have the largest number of transporters whereas the α cyanobacteria or oceanic species have lower number of transporters. The following sections will briefly discuss these systems (Price et al., 2008).

BicA is a high flux, low to medium affinity sodium dependent HCO_3^- transporter. It belongs to the SulP family (Price and Howitt, 2011) and is probably a Na^+/ HCO_3^- symporter since its stoichiometry is unknown. BicA belongs to the family of SulP/SLC26 transporters (Price et al., 2004) whose homologs are present in the genomes of both β and α cyanobacteria. Gain of function mutants in freshwater show that this transporter alone could conduct sodium dependent HCO_3^- uptake activity to reach half maximal HCO_3^- uptake. BicA is upregulated in low CO_2 in *Synechococcus* PCC7002 and has low expression in high CO_2. However, it is constitutive in PCC6803 (Price et al., 2004). In oceanic strains its expression levels are still undetermined. Topology predictions estimate 12 transmembrane domains and a 60kDa protein in PCC7002. The C terminus has a hydrophilic region of 102 amino acids facing the cytoplasm which is involved in allosteric transport regulation. BicA has a STAS domain since it belongs to SulP family and this domain has regulatory function in several mammalian homologs (Ko et al., 2004; Price et al., 2008). The vital difference between BicA and other transporters in this family is the cytoplasmic loop between helix 8 and 9.

SbtA is another inducible Na^+ dependent HCO_3^- symporter which is mostly found in prokaryotes. It's a single unit transporter and has an affinity for HCO_3^- of around 2 µM

at pH 9.3 and supports only a low flux rate in *Synechococcus* PCC7002 (Price et al., 2004). Despite a low flux rate the affinity of SbtA is strong which is nearly 2-5 µM.

Nearly 1mM Na^+ is needed to reach half max HCO_3^- uptake for SbtA (Shibata et al., 2002). It is suggested that SbtA has Na^+/ HCO_3^- symporter activity such that Na^+ is directed inwards and is maintained by a Na^+/H^+ antiporter (Espie and Kandasamy, 1994). Flux studies and gain of function mutants might be able to throw more light on these speculations. Under limited Ci, SbtB, a small gene downstream of SbtA is co-expressed (Wang et al., 2004). It was recently found to be a dark inhibitor of SbtA which reduces Ci uptake (Du et al., 2014). It binds to SbtA to reduce Ci uptake and acts in a post translational manner. The complex size of isolated SbtA from cytoplasmic membranes is 140kDa (Zhang et al., 2004) and suggests the existence of a functional tetramer. SbtA is also expressed under limited CO_2 levels and as per topology results from *Synechococcus* PCC7942, the protein is 39.5kDa in size with 9 transmembrane domains. There are two central hydrophilic regions of 60 and 34 amino acids which face the cytoplasm and may be involved in allosteric regulation of SbtA (Price et al., 2008). Several homologs of SbtA have been found in β cyanobacteria especially in *Synechocystis* PCC6803 (Shibata et al., 2002) and *Synechococcus* PCC7002 (Price et al., 2004) but only weak isoforms have been discovered in α cyanobacteria.

BCT1 is the third HCO_3^- transporter which has a high affinity for HCO_3^- and belongs to an ATP binding cassette (ABC) transporter family, proteins that are ATP energized (Omata et al., 1999; Higgins, 2001). BCT1 has been characterized in *Synechococcus* PCC7942 and close homologs have been found in ten other species. This transporter is encoded by cmpABCD, an operon expressed under Ci limitation as well as high light

stress (Omata et al., 1999; Price et al., 2008). BCT1 in gain of function mutants has a high affinity for HCO_3^- at 15μM and a medium rate of flux (Omata et al., 2002). BCT1 is a complex of four subunits and appears to be a uniporter for HCO_3^-. CmpA is a 42kDa lipoprotein bound to the periplasmic face of plasma membrane where it binds HCO_3^- or acts like a mobile carrier. It's the most abundant component of BCT1 which is known to collect the substrate and then pass it on to the transporter (Higgins, 2001). The crystal structure reveals a calcium binding site as a co-factor but no information is available if calcium plays a role in the actual HCO_3^- transport. CmpB is a membrane protein which is a hydrophobic dimer within the membrane. Thus, by analogy to other members of the ABC transport group it forms a transport path in the membrane. CmpC and CmpD have ATP binding sites and are present on the cytoplasmic face of CmpB. CmpC has additional domains unlike CmpD which may help in allosteric regulation of BCT1.

Genome data comparison shows BCT1 is only present in β cyanobacteria and not in marine organisms (Badger et al., 2005). The reason for this lies in the strategy of using a sodium inward gradient for energizing uptake rather than using ATP as a source of energy (Bryant, 2003). However coastal *Synechococcus* WH5701 (draft assembly coordinated by Dr Dave Scanlan and the Moore Foundation, www.moore.org) may change this idea since it has homologs for all transporters for HCO_3^- as found in β cyanobacteria. *Synechococcus* WH5701 survives in both freshwater and saltwater and thus handles Ci limitation conditions well.

NDH-I_4 and NDH-I_3 are the two CO_2 uptake systems in cyanobacteria. NDH-1_4 is located on the plasma membrane and NDH-1_3 is located on the thylakoid membrane. I_3 is inducible under limiting Ci and has high affinity for CO_2 uptake (Zhang et al., 2004).

Both are uptake systems for CO_2 such that CO_2 entering the cell can be converted to HCO_3^- so it does not leak out and in turn accumulates as Ci. These are essentially facilitators of Ci into the cell and are based on NADPH dehydrogenase complexes. There are several single copy genes in the genome of *Synechocystis* 6803 for CO_2 uptake and some which have three to six homologs. Initial data indicates the gene clusters needed to accumulate Ci are : ndhAIGE, ndhCJK, ndhH, ndhB and ndhL. There are several copies of ndhD and ndhF. ndhD1/D2 gene products with ndhF1 forms a respiratory NDH-1 complex which oxidizes NADPH/NADH and reduces plastoquinone (Maeda et al., 2002). This enables cyclic electron transport around PSI. NdhD3/D4 proteins, and NdhF3/F4 form parts of the NDH-1 complex which aids CO_2 uptake by converting CO_2 to HCO_3^- (Shibata et al., 2001; Maeda et al., 2002). An Mnh cluster maybe present in cyanobacteria that helps in sodium dependent HCO_3^- uptake involved in SbtA and BicA. An Mnh cluster expressed as a bicA operon may be involved in HCO_3^- uptake efficiency to improve photosynthesis (Woodger et al., 2007). There are also two CO_2 uptake systems in place as part of the NDH-1 complex called ChpX and ChpY (Maeda et al., 2002) which hydrates CO_2 and forms HCO_3^-.

Carboxysomes

Carboxysomes are proteinaceous structures in the cytosol of cyanobacteria as well as several anoxygenic phototrophic bacteria and archaea chemolithotrophs (Raven, 2010). Rubisco is localized in the carboxysome along with carbonic anhydrases which convert incoming HCO_3^- to CO_2 which can be fixed by Rubisco (Price et al., 1998). HCO_3^- apparently can diffuse inside the carboxysome which has selective anion pores. Despite low levels of carbonic anhydrase (CA), there is enough to convert the

HCO_3^- into CO_2 within the carboxysomes. β cyanobacteria have β Carboxysomes with Form IB Rubisco and α cyanobacteria have Form IA Rubisco in α Carboxysomes. These have evolved in parallel but some proteins in the shell are related. There is a ccmKLMNO operon in *Synechococcus* PCC7942, which has a β carboxysome, which along with the carboxysomalCcaA helps in carboxysome functioning. The carboxysome also participates in a CO_2 recapture system where the protein shell acts as a barrier to CO_2 leakage (Kaplan and Reinhold, 1999). Carboxysomes have a shell made of at least four types of polypeptides which are known as Cso1 proteins. Homologs have been found in β Carboxysomes and are called ccmK, m and O. Generally, these proteins and their homologs aid in carboxysome formation. Cryo-electron tomography reveals an icosahedral shape of the carboxysome which looks like a flattened hexagon (Schmid et al., 2006). α carboxysomes have CsoS2 (80kDa) and CsoS3 (60 kDa) where the latter is a CA that not only dehydrates CO_2 but also maintains the structure of carboxysomes (Price et al., 2008). In β carboxysomes there are CcmM (70kDa), CcmN (26kDa) and CccA which is a carboxysomal β CA (Price et al., 1998; Cannon et al., 2001; So et al., 2002). CcmM has repeat motifs on its C terminal end which are homologous to Form 1B Rubisco small subunit (SSU) (Price et al., 1998; So et al., 2002). It is a structural component of carboxysomes (Price et al., 1998) and there is evidence of interaction between holoenzymes and CcmM which helps to form the icosahedral shape in carboxysomes (Price et al., 2008). There is no direct evidence but some possibility that CsoS2 may play a similar role in carboxysomes. A newly discovered protein CcmK can assemble hexamer flat plates of 7 nm and larger sheets, aiding in carboxysome organization. This protein and minor proteins like CcmL, N and O form the outer

carboxysome coat with CcmM and Rubisco associations as mentioned above (Kerfeld et al., 2005). The negative ion permitting coat of the carboxysome is formed by CcmK which has a small hole in the center of each plate that allows HCO_3^-, PGA , RuBP and OH^- to pass (Kerfeld et al., 2005). It is speculated that due to its homologous similarities to CcmK, CsoS1 proteins may be performing the same role in α carboxysomes.

Cyanobacterial Carbonic anhydrase (CA)

CsoCA is the sole CA in α carboxysomes and belongs to the ε group of CA. CsoCA is also an integral part of the carboxysome shell and might traverse the shell (Baker et al., 2000; Heinhorst et al., 2006). This speculation arises from the fact that the protein could not be stripped from the shell using detergents or high salt washes. Urea treatment releases CsosCA from the shell (Cannon et al., 2001). Recombinant CsoSCA is a heterodimer of 120kDa with one zinc ion per monomer. Removal of this ion results in loss of activity. K_{cat} values for this CA for hydration and dehydration reactions , which depend on pH, are within the range of β CAs (Heinhorst et al., 2006). Insertional mutants for CsoSCA require elevated CO_2 to survive (Dou et al., 2008) but do not have any structural difference in the carboxysome microcompartment from the wild type. The V_{max} of the purified carboxysomes from the mutant is not different from the wild type but the $K_{0.5}$ is three times higher than the wild type. Adding recombinant CsoSCA did not restore wild type levels of activity in the mutant, which suggests that CO_2 inside the carboxysome has to be increased to restore activity. This reinforces the idea of an anti-leakage protein shell in the carboxysome. Thus, the carboxysomal CA is needed to maintain a high concentration of CO_2 around Rubisco for efficient cell growth.

CcaA is a component of β carboxysomes in *Synechococcus* PCC7942 and *Synechocystis* PCC6803 (Fukuzawa et al., 1992; Yu et al., 1992; So et al., 2002). Despite being separated from the carboxysomal cluster of genes, mutants for this CA need high CO_2 to grow and indicates that the gene is essential for CO_2 fixation (Kerfeld et al., 2005). High CO_2 requiring mutants of *Synechococcus* PCC7942 have been rescued using genomic DNA from CcaA gene which has homology to β CA in plants and *E.coli*. Antibodies raised against the recombinant homolog of CcaA in *Synechocystis* PCC6803 could localize the enzyme in the carboxysome. Mutants of the enzyme were found to have carboxysomes devoid of CA activity (So and Espie, 1998). Such mutants also had lower Ci affinity and lower maximum catalytic rates than the wild type (So et al., 2002). Ci accumulation rates, however, were not affected indicating that utilization of the accumulated HCO_3^- was disrupted, as is usual in a carboxysomal CA mutant (Fukuzawa et al., 1992; So and Espie, 1998). CcaA has several orthologs with highly conserved residues except for the C terminus of the protein, which harbors residues not found in other β CAs. This region is also important for intramolecular interactions and catalytic activity of the enzyme (So et al., 2002).

CcmM is the second putative β carboxysomal CA, which is always found in a gene cluster encoding carboxysomal shell proteins as well as CcmN, a gene essential for the formation of the carboxysome. Mutants for this CA do not have a carboxysome and have a high CO_2 requiring phenotype (Ludwig et al., 2000; Berry et al., 2005; Woodger et al., 2005; Emlyn-Jones et al., 2006).The N terminus of CcmM is homologous to γ-CA in archaea (Price et al., 1992). (Cot et al., 2008) indicated that recombinant CcmM can bind to HCO_3^- and a strain lacking CcaA in *Nostoc PCC7120*

still contains CA activity which might arise from CcmM. CcmM has two isoforms in *Synechococcus* 7942- 58 kDa and 35 kDa. The 58 kDa form essentially encompasses the predicted length of CcmM and binds to CcaA but the 35 kDa form has only Rubisco like subdomains and binds Rubisco (Price et al., 1998; Long et al., 2010) . Cells without M58 have no CcaA electron dense structures and a high CO_2 requiring phenotype which suggests that M35 is needed to package Rubisco but M58 is essential for forming functional carboxysomes (Long et al., 2010). Yeast 2 hybrid proves that C terminus of CcmM and CcaA interact in *Synechococcus* PCC7942. There is also evidence which shows that above mentioned complexes include Rubisco and CcmN (Cot et al., 2008). The orientation of the CA has been proposed to be similar to CsoSCA in α carboxysomes such that incoming HCO_3^- is dehydrated to CO_2 simultaneously (Long et al., 2007; Cot et al., 2008). Hence its proposed that there is a multiprotein complex for dehydration of HCO_3^- in β carboxysomes. Other than binding to incoming HCO_3^-, CcmM also recruits parts of the complex and regulates access to the interacting CcaA.

CCM in *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a popular model organism due to its quick doubling time, sequenced genomes (nuclear, mitochondria and chloroplast) (Grossman et al., 2007; Merchant et al., 2007) and heterotroph growth abilities (Harris et al., 2009). The CCM In Chlamydomonas was first discovered in 1980 by Badger. It is one of the most well studied CCMs in eukaryotic algae. It has several similarities with the cyanobacteria CCM including the ability to accumulate HCO_3^- , packing Rubisco in a compartment where CO_2 is dehydrated from HCO_3^- and fixed by Rubisco (Moroney et al., 2011). The overall biochemistry of Chlamydomonas photosynthesis is C3, unlike

CAMs or C4 plants which convert the incoming HCO_3^- to a C4 acid. Loss of more than one component of the CCM causes a high CO_2 requiring phenotype in the mutant and poor affinity for inorganic carbon (Ci). Several CCM components are induced in the Chlamydomonas CCM when it is switched from high CO_2 (5% CO_2) to low CO_2 (0.01% CO_2). It takes nearly 4 hours for the CCM to be induced in Chlamydomonas and for Ci (CO_2 and HCO_3^-) to increase 20 fold (Moroney et al., 2013). The $K_{0.5}$ of cells grown in high CO_2 is 10-1000 times higher than for cells grown at low CO_2 cells (Moroney et al., 1985) which is due to the fact that the CCM is not induced in a high CO_2 environment. In low CO_2, active uptake of Ci occurs, which is an energy intensive process (Spalding et al., 1983). The energy is probably obtained from cyclic electron transport, which also increases during limiting CO_2 conditions. The components of the *Chlamydomonas* CCM will be described in detail in the following sections as shown in fig.1.5.

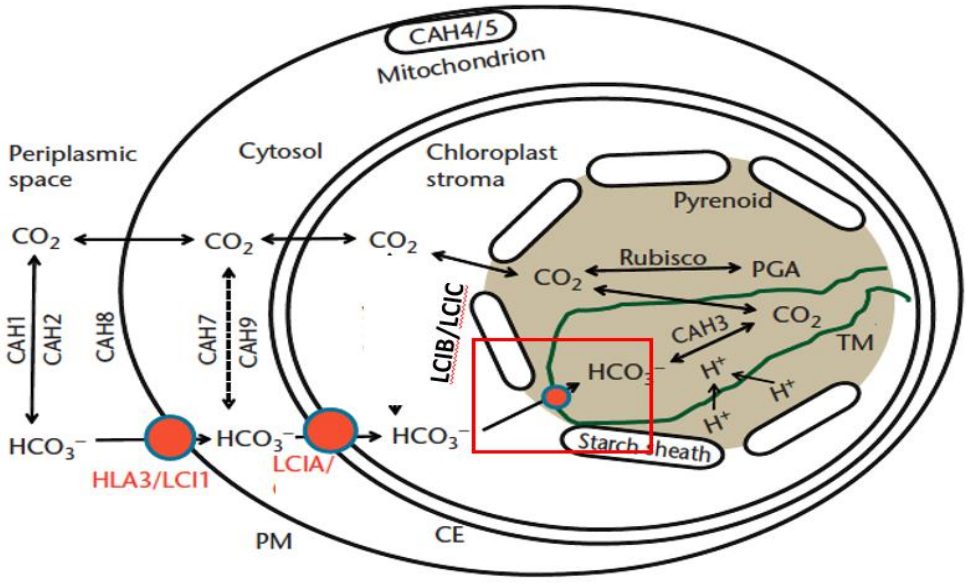


Figure 1.5. Schematic representation of CCM in Chlamydomonas (Mukherjee, 2013). Ci transporters are labelled in red with red dots indicating areas of HCO_3^- uptake, black arrows show the entry of CO_2, white discs structures show the starch sheath surrounding the pyrenoid where Rubisco and CAH3 are localized.

Pyrenoid

Pyrenoids are electron dense organelles in chloroplast of most eukaryotic algae and the number and shape of these organelles have been used to classify the Chlamydomonas genus before 18S rRNA sequences were made available (Harris et al., 2009). Chlamydomonas has a starch sheath enclosing a, single prominent pyrenoid which is the location of 90% of the Rubisco in the cell (Ramazanov et al., 1994; Rawat et al., 1996; Morita et al., 1997; Borkhsenious et al., 1998).This sheath makes the pyrenoid boundary more defined and distinct in limited CO_2 conditions (Ramazanov et al., 1994). When cells are switched from high to low CO_2 conditions, the Rubisco starts to redistribute and accumulate in the pyrenoid within 2 hours of exposure to limited CO_2 (Borkhsenious et al., 1998). Rubisco activase, the enzyme responsible for activating Rubisco by removing inhibitory sugar phosphates, is also present in the pyrenoid (McKay et al., 1993). Rubisco distribution analysis via FRAP and cryoelectron microscope experiments also reveal that the pyrenoid is a fluid crystal structure (Rosenzweig et al., 2017). In fact there are no proteins in the pyrenoid larger than 78kDa which are part of the pyrenoid (Rochaix, 2017). The relocalization of Rubisco in high CO_2 from pyrenoid to stroma or during dark shifts the pyrenoid matrix to a soluble phase from aggregated phase (Rosenzweig et al., 2017).

RbcS has been demonstrated to be essential for pyrenoid formation (Meyer et al., 2012). In fact using large subunits from Chlamydomonas with RbcS from plants created a strain that had similar kinetic properties as the WT Chlamydomonas Rubisco but lacked the ability to grow in low CO_2 and did not have a pyrenoid (Genkov et al., 2010). Another gene important for pyrenoid formation is CIA6 (Ma et al., 2011). *cia6* had

normal levels of Rubisco but had a disrupted pyrenoid and reduced Ci affinity. The *cia6* mutant also had higher levels of chlorophyll than the WT strain (Ma et al., 2011). The CIA6 gene is a lysine methyltransferase and contains a SET domain but no *in vitro* methyltransferase activity was found in *cia6* (Ma et al., 2011). A recent finding characterized a pyrenoid protein that functions as a Rubisco linker protein called EPYC1 (Essential Pyrenoid Component) (Mackinder et al., 2016). The *epyc1* mutant has a disrupted CCM with reduction in the assembly of Rubisco in the pyrenoid. This protein has four nearly identical repeat regions which forms a complex with Rubisco (Mackinder et al., 2016). A recent interactome study revealed 38 novel components of the pyrenoid via florescent protein tagging along with affinity mass spectrometry (Mackinder et al., 2017). The aforementioned interactome study also revealed that the pyrenoid has a plate like layer, mesh and a punctuate layer (Mackinder et al., 2017). Chlamydomonas has a thylakoid tubule network that penetrates the starch sheath and the pyrenoid itself (Griffiths, 1970). *In situ* cryo-electron tomography (Engel et al., 2015) showed that the pyrenoid and thylakoid stacks were connected by cylindrical pyrenoid tubules. Inside the tubules there are mini tubules which run parallel and whose membranes are derived from adjacent bundled thylakoid membranes. These mini tubules act as a passageway between the stroma and might facilitate diffusion of metabolites. The thylakoid tubules traversing the pyrenoid contain a vital carbonic anhydrase CAH3 which converts incoming HCO_3^- to CO_2, to be fixed by the Rubisco inside the pyrenoid to 3-PGA. More details on carbonic anhydrases will be provided in the following section.

Carbonic anhydrases (CA)

The α, β, ϒ and ζ classes of CAs are present in Chlamydomonas. CAH1 and CAH2 are periplasmic α CAs (Fujiwara et al., 1990; Fukuzawa et al., 1990). They are similar to each other with only a few amino acid differences in the protein sequence. CAH2 may have resulted from a gene duplication event and is not highly expressed compared to CAH1. CAH1 has two small and two large subunits bound by disulfide bridges. CAH1 is also processed posttranslationally such that it is directed to the ER after the cleavage of the leader sequence. Additionally, it is glycosylated and the mature protein is then directed to the periplasmic space. CAH2 is also processed similarly such that the leader and an internal region is cleaved (Ishida et al., 1993). CAH1 is induced in large amounts under low CO_2 but the protein is so abundant that it is not broken down completely over if the cells are switched to high CO_2 from low CO_2 to up to 4 days. A mutant for CAH1 did not show any significant physiological differences compared to the WT strain. CAH7 and CAH8 are β CAs which also are very similar to each other in sequence with unusual C termini sequences (Ynalvez et al., 2008). The C termini are hydrophobic with transmembrane helices. CAH7 has not been precisely localized but CAH8 is localized to the periplasm along with CAH1 and CAH2 as per immunogold labelling. The presence of 3 CAs in the same location might explain the redundancy of their functions. This might also explain why photosynthesis is largely unaffected in the mutant ca1. CAH7 and CAH8 are constitutive CAs and there is no difference in expression in low or high CO_2 grown cells for these genes (Mitra et al., 2005). CAH8 and CAH7 are both fairly abundant in expression if not as abundant as CAH1.

Mitochondrial CAs CAH4 and CAH5 are β CAs which are both strongly upregulated when high CO_2 grown cells are shifted to low CO_2 for 4 hours (Eriksson et al., 1995; Eriksson et al., 1996). They are 95% identical to each other on the gene level and their proteins differ by a single amino acid (Eriksson et al., 1996). It is speculated that like CAH1 and CAH2 these are also a result of gene duplication. CAH4and CAH5 exist as an inverted repeat on chromosome 5. Due to lack of a mutant so far, the role of CAH4/5 have not been elucidated in Chlamydomonas. However, it is speculated that these CAs may play a role in anaplerotic reactions in the mitochondria, retain CO_2 or regulate the pH of the cell (Raven, 2001; Giordano et al., 2003).

Another βCA is CAH9 whose sequence diverges from other CAs of this family. It is the least characterized and is predicted to be localized to the cytoplasm(Moroney et al., 2011). The expression levels of CAH9 are very low which makes it unlikely to be a part of Ci acquisition of retention like the other CAs (Miura et al., 2004; Mitra et al., 2005). CAH6 is another constitutive βCA which is localized to the flagella and is part of the flagellar proteome (Pazour et al., 2005; Mackinder et al., 2017). It is speculated that CAH6 in the flagella may participate in sensing low levels of CO_2 because Chlamydomonas is chemotaxic to HCO_3^- (Choi et al., 2016) and CAs participate in carbon sensing (Hu et al., 2010).

CAH3 is localized to the thylakoid lumen (Karlsson et al., 1998) and due to an acidic luminal pH most of the inorganic carbon entering as HCO_3^- is converted to CO_2 by CAH3. This CO_2 is then released to the Rubisco inside the pyrenoid to fix CO_2. It is an abundant constitutive CA and strains where CAH3 is inactivated are very sick in low

CO_2 (Spalding et al., 1983; Moroney et al., 1986). The mutants for CAH3 also have reduced affinity to Ci which proves that CAH3 is required for the CCM to be functional. LCIB and LCIC which were formerly known as chloroplast proteins which aided in CO_2 acclimation in limiting conditions are a new group of Θ CAs (Kikutani et al., 2016). LCIB and LCIC mutant die in air and have a severely reduced Ci affinity compared to WT (Wang and Spalding, 2006, 2014). It has been hypothesized that LCIB/C participate in unidirectional hydration of CO_2 to HCO_3^- to supply HCO_3^- to the thylakoid (Duanmu et al., 2009; Wang and Spalding, 2014). Both the proteins form a complex, which under high CO_2 conditions remain dispersed throughout the stroma but form a sheath like localization pattern around the pyrenoid in low CO_2 conditions (Yamano et al., 2010; Wang and Spalding, 2014). This leads to a speculation that an additional function for LCIB/C might be recapture of CO_2 back to the pyrenoid especially in the mutant for *cia6* where LCIB levels are lower than the WT strain (Ma et al., 2011).

γ CAs are CAG1, CAG2and CAG3 which show similarity to higher plant γ CAs are all formed on different chromosomes in Chlamydomonas. CAGs are abundant proteins which may be involved in complex I of mitochondria as they belong to the mitochondrial proteome (Moroney et al., 2011) . CAG1, CAG2 and CAG3 have not been characterized further.

Ci transporters

There are several transporters in Chlamydomonas which bring in HCO_3^- . HLA3 and LCI1 are on the plasma membrane. HLA3 comes from the acronym High Light Activated genes. It is a putative ATP-binding cassette (ABC) type transporter of the multidrug-resistance-related proteins (MRP) subfamily (Im and Grossman, 2002). HLA3

is induced by light and limited CO_2. HLA3 is controlled by a transcription factor CIA5, which controls most CCM genes (Fukuzawa et al., 2001; Miura et al., 2004; Fang et al., 2012). Knocking down HLA3 in LCIB mutant causes a sick on low CO_2 phenotype with reduced Ci affinity at high pH (Duanmu et al., 2009). If HLA3 is knocked out with the chloroplast envelope Ci transporter NAR1.2/LCIA Ci uptake is markedly reduced (Yamano et al., 2015). HLA3 when expressed in Xenopus oocytes has shown moderate uptake of HCO_3^- (Atkinson et al., 2016). LCI1 is another plasma membrane transporter (Burow et al., 1996; Ohnishi et al., 2010). It is also controlled by CIA5 and LCR1, another transcription factor that controls several CCM genes (Yoshioka et al., 2004). Overexpressing LCI1 in the *lcr1* mutant increases Ci affinity and promotes uptake of Ci (Ohnishi et al., 2010). LCI1 is encoded by an orphan gene since there are no known homologs of this gene in other organisms. Some structurally similar membrane proteins have been found in other Chlamydomonas genomes. The interactome study of 2017 found HLA3 and LCI1 in a complex with strong interactions (Machingura et al., 2017). They also formed a complex with ACA4, a P-type ATPase/cation transporter (Mackinder et al., 2017). This ATPase might help in HCO_3^- uptake by maintaining a H^+ gradient. HLA3 also interacts with a adenylate/guanyl cyclase which are known to sense inorganic carbon in several taxa (Tresguerres et al., 2010).

LCIA/NAR1.2 is a chloroplast membrane transporter and a single mutant causes reduction in Ci affinity and growth only at high pH (Wang and Spalding, 2014). However if knocked out along with LCIB (Wang and Spalding, 2014) or HLA3 (Yamano et al., 2015) there is a reduction in growth at regular pH and Ci uptake. RSecent studies indicate that HLA3 mRNA levels are affected by the absence of LCIA but the reverse is

not true (Yamano et al., 2015). The molecular mechanism of LCIA is elusive. LCIA belongs to a family of nitrate assimilation-related (NAR) proteins but its controlled by low CO_2 (Miura et al., 2004). LCIA is a homolog of the bacterial formate transporter (FocA) with five amino acid residues that correspond to the pore of FocA (Wang et al., 2009). FocA resembles the structure of aquaporins and transports formate as a channel. Similarly it is speculated that LCIA is also a channel. Ci affinity increases in LCIA overexpressed strains and it might be functioning as a channel that causes an increase in Ci conductance with minimum concentration gradient and without a prominent increase in Ci in the cytoplasm (Yamano et al., 2015).

CCP1/CCP2 are two potential transporter candidates localized in mitochondria with similarities to a mitochondrial protein carrier superfamily (Spalding and Jeffrey, 1989; Geraghty et al., 1990; Chen et al., 1997; Atkinson et al., 2016). CCP1/2 knockdowns did not show any difference in Ci affinity despite exhibiting a sick on low CO_2 phenotype like other CCM mutants (Pollock et al., 2004). These proteins aid Ci transport but do not have a direct role in taking up or accumulating Ci.

There is no evidence of a thylakoid HCO_3^- transporter. However, several studies have speculated the involvement of one or several transporters in the thylakoid (Moroney and Ynalvez, 2007; Spalding, 2008) such transporters will transport HCO_3^- into the thylakoid lumen to be dehydrated by CAH3. This will be the topic of chapter 4 in this thesis.

CCM regulation in Chlamydomonas

A protein interactome showed the interaction of several CCM proteins with each other and probably identified several new ones(Mackinder et al., 2017). Other than the

CO_2 concentration, light and circadian rhythm also affect CCM gene expression in Chlamydomonas. Lack of light reduces Ci affinity and CCM gene expression (Mitchell et al., 2014). However before the dark period ends CCM genes HLA3, LCIA and LCIB are upregulated and CAH3 and Rubisco localizes to the pyrenoid (Rawat et al., 1996; Mitchell et al., 2014).

RNA-seq has revealed that CCM genes are induced by low CO_2 levels and controlled by CIA5/CCM1, a transcription factor, for the most part (Fang et al., 2012). CIA5 is a well studied transcription factor that controls several CCM genes and the *cia5* mutant has very low Ci affinity due to lack of induction of the known HCO_3^- transporter-HLA3, LCI1 or LCIA (Moroney et al., 1989; Fukuzawa et al., 2001; Xiang et al., 2001; Miura et al., 2004; Fang et al., 2012). The RNAseq study from 2012 (Fang et al., 2012) also shows genes unaffected by CO_2 levels are controlled by CIA5. This leads to the speculation that CIA5 may regulate genes other than CCM genes. CIA5 has two Zn binding domains at the N terminus and is a hydrophilic protein. On the C terminus it has several putative phosphorylation sites and a glycine repeat region (Fukuzawa et al., 2001; Miura et al., 2004). CIA5 has constitutive expression with respect to CO_2 levels but Zn deficiency causes CIA5 upregulation maybe due to its zinc binding ability. The Zn binding motifs indicate that it's likely to be DNA binding transcription factor (Xiang et al., 2001) and regulates downstream genes.

LCR1 is another transcription factor which was identified when the mutant, lcr1 was identified (Yoshioka et al., 2004). An Myb type transcription factor, LCR1 regulates a subset of genes like CAH1, LCI1 and LCI6 (Yoshioka et al., 2004). It itself is upregulated by CIA5 and low CO_2. CAH1 is transiently expressed in lcr1 in low CO_2

which leads to the conclusion that constant expression of CAH1 requires LCR1 by binding Cis binding elements in CAH1 promoter (Yoshioka et al., 2004).

A recent study found a calcium sensor protein (CAS) which adds another layer of control to some CO_2 induced genes in the CCM (Wang et al., 2016). CAS was identified as a calcium binding protein when H82, the mutant for CAS was characterized. This protein is needed to maintain the expression of at least 13 nuclear encoded genes including but not limited to HLA3 and LCIA (Wang et al., 2016). It was found that expression of both HLA3 and LCIA was reduced in H82 but the Ci affinity could not be rescued by the expression of both genes. CIA5 and CAS do not need each other to express which suggests that both the transcription factors function in parallel. CAS is transported to the pyrenoid under low CO_2 and light conditions from thylakoid membranes. Another follow up study reveals that CAS forms a mesh like structure in high CO_2 in the chloroplast but the signal forms a wheel like structure in the pyrenoid after2 h of low CO_2 induction and at 12 h moves to the pyrenoid (Yamano et al., 2018). This leads to the speculation that CAS moves in response to CCM induction.

CHAPTER 2
MATERIALS AND METHODS

Cell culture and growth

Chlamydomonas culture conditions were according to the conditions used previously (Ma et al., 2011). The D66 strain (*nit2*$^{-}$*, cw15, mt*$^{+}$) is from Dr. Rogene Schnell (University of Arkansas, Little Rock) and CMJ030 (CC-4533; *cw15, mt*$^{-}$*)* and *bst3* (BST3 knockout LMJ.RY0402.089365) were obtained from the CLiP collection at the Chlamydomonas culture collection (Zhang et al., 2014). Tris-Acetate-Phosphate (TAP) and Minimal (MIN) media (acetate free) were made according to Sueoka (Sueoka, 1960). TAP and MIN plates for growth were made by adding 1.2% (w/v) agar. Colonies from TAP plates were used to inoculate 100 mL TAP liquid for mixotrophic growth. Growth in liquid TAP was under continuous illumination (100 µE m^{-2} s^{-1}) and shaking for 48 h. Early log phase TAP cultures were harvested and washed with MIN media, followed by resuspension in MIN media and bubbled with high CO_2 (5% [v/v] CO_2 in air) to reach OD_{730} between 0.2 and 0.3 (~2-3 x10^6 cells mL^{-1}). This is followed by CCM induction when the cells were transferred to low CO_2 (0.04% [v/v] CO_2 in air) bubbling for 12 h. For growth curves early log phase TAP cultures were diluted to OD_{730} 0.01 in acetate free or replete media and grown in low CO_2 (0.04% [v/v] CO_2 in air in under continuous illumination (150 µmol m^{-2} s^{-1}).

Photosynthetic assays

100mL TAP was inoculated with colonies of Chlamydomonas cultures for 48 h in the light (100 µmol m^{-2} s^{-1}) to logarithmic phase. Cells were then washed with MIN media and transferred to MIN media, and then bubbled with 5% CO_2 until they reached a cell

density OD_{730} = 0.2-0.3 ($3x10^6$ cells mL^{-1}). The cells were then bubbled with low CO_2 (0.04%) in the light for 12 h to induce the CCM. The external Ci was estimated according to (Ma et al., 2011). Briefly, cells equivalent to 100µg chlorophyll were suspended in 4 ml HEPES-NaOH buffer (pH 7.4) or 25 mM EPPS-NaOH buffer (pH 8.4 and 7.8) that had been bubbled with nitrogen gas. An O_2 electrode chamber (Rank Brothers, Cambridge UK) illuminated at 300 µmol $m^{-2}s^{-1}$, was used to deplete DIC until no net oxygen exchange is seen. Increasing concentration of $NaHCO_3$ was then injected into the depleted cells. $K_{0.5}$(Ci) was then calculated as DIC concentration needed for half maximal rate of oxygen evolution. For respiration assays the above process was carried out in the absence of light and in 4 ml HEPES-NaOH buffer (pH 7.4) only.

Inorganic carbon uptake

Silicone oil centrifugation was used to measure intercellular concentration of dissolved Ci as per (Moroney et al., 1985). Briefly, cells were centrifuged and suspended at 25µg Chl mL^{-1} density in Ci depleted 25 mM EPPS-NaOH (pH 7.8 or 8.4) and incubated in the light until net O_2 evolution was zero. Cells were maintained in the light until used. 300 µL of Ci depleted cells were then centrifuged in tubes containing 25 µL of 1 M glycine (pH 10) with 0.75% (w/v) SDS overlaid with 75 µL of Dow Corning AR 20 silicone oil. Assays were performed at 25°C in 200 µmol m^{-2} s^{-1} light in a Beckman Microfuge B. Ci uptake was initiated by adding either 3 µl of 25mM (pH 7.8) or 50mM (pH 8.4) $NaH^{14}CO_3$ followed by the indicated time of illumination (between 15 and 120 sec at 150 µmol m^{-2} s^{-1} light). The reaction was terminated by a 15 second centrifugation in a microfuge B (Beckman). Internal Ci was calculated using the difference between total and acid stable

^{14}C in the pellet and corrected for cell volume as previously described (Machingura et al., 2017).

Fluorescence protein tagging

The *BST1-3* genes were cloned and transformed into Chlamydomonas strain CC-4533 as in Mackinder et al (2017). Briefly, the open reading frames of *BST1-3* genes were PCR amplified from genomic DNA and cloned into pLM005 with C-terminal Venus-3xFLAG and a *PSAD* promoter through Gibson assembly. For transformation, wild type cultures were grown to mid-log phase and concentrated to $2x10^8$ cells mL^{-1}. The suspension was mixed with the constructed plasmid linearized by *EcoRV* prior to electroporation. Suspension was then plated on TAP paromomycin (20 µg mL^{-1}) for selection. Fluorescent colonies were identified using a Typhoon 8610 scanner. Laser settings for Venus were 532nm for excitation and 555/20 for emission, and chlorophyll autofluorescence was excited at 633nm with 670/30 emission.

Confocal microscopy

Fluorescent colonies were identified grown heterotrophically in TAP medium to reach mid-log phase. Cultures were then harvested and re-suspended in Tris-minimal medium overnight prior to imaging. Images were captured with Laser-scanning microscope LSM880 (Zeiss) equipped with an Airyscan module using an x63 objectives with 1.4 NA. Argon laser at 514 nm and 561 nm were used for excitation of Venus and chlorophyll respectively. Filters were set at 525 – 550 nm for the Venus emission and 620 – 670 nm for chlorophyll emission.

Generation of RNAi constructs

Artificial microRNA construct for the knock-down of the BST proteins were made using the protocol of(Molnar et al., 2009). Briefly the Web MicroRNA Designer (WMD3) website (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was used to design oligos complementary to the region of identity of the three BST coding sequences. Two independent constructs were designed and cloned in to the pChlamyRNA3int plasmid obtained from the Chlamydomonas resource center. Appendix I has the list of oligos used for the miRNA constructs.

Electroporation

Transformations to generate RNAi knockdown strains and large scale insertional mutants described in chapter 4 and 5, D66 was mutagenized with the plasmid containing the insert for the specific gene using the electroporation method described in (Shimogawara et al., 1998) with some modifications from the Invitrogen transformation buffer kit. In brief, cells were grown on TAP liquid as mentioned above and CCM induced by bubbling for 12 h in MIN media under continuous light (400 µE m^{-2} s^{-1}). Cells were harvested and resuspended in buffer at a concentration of 2 x 10^8 cells mL^{-1}. DNA (500ng per cuvette) was added to 300 µl of cells and incubated on ice for 30 minutes in an electroporation cuvette. This is followed by electroporation in Gene Pulser II electroporator (Bio-Rad) with a modified 25-mF capacitor and a shunt resistor of 330 V. Cells then recovered in TAP sucrose (40 mM) in dark and were then plated out on TAP paromomycin plates. Colonies were picked after plates were grown in dim light for seven days. The RNAi mutants were grown in high CO_2 in continuous light for seven days.

Mutant isolation and phenotypic screen

The pChlamyRNA3int plasmid carrying the *AphVIII* gene that confers paromomycin resistance ($para^R$) was transformed into D66 by electroporation (Shimogawara et al., 1998). Transformants were selected on TAP agar media containing the antibiotic paromomyCin (4 µg mL^{-1}; Invitrogen). Resistant strains were then screened for "sick on low CO_2" phenotype by replica plating them on MIN plates. These were then placed in a high CO_2 chamber (5% [v/v]) CO_2 in air and a low CO_2 chamber (0.01% [v/v] CO2 in air) with continuous illumination (100 µmol m^{-2} s^{-1}) for 7 days. Growing cells were suspended in liquid MIN medium to same cell density for spot tests (OD730 =0.1, 0.05 and 0.025) and 15µL was spotted onto MIN plates. These plates were placed in high, ambient and low CO2 chambers for 7 days. The CO_2 concentration was measured using an Environmental Gas Monitor (EGM-4, PP systems, Amesbury, MA).

For the generation of the mutant A144 in chapter 5, D66 was used as the wild type which was mutagenized by pSL18 using electroporation as previously described by (Shimogawara et al., 1998). TAP paromomyCin (5 µg·mL^{-1}, Invitrogen, Carlsbad, CA) plates were used for selecting transformants. A CCM deficient phenotype was screened using the same process as mentioned above.

Confirmation of flanking region

Primers specific to the *BST3* and *EF3* gene were made complementary to the last exon to span the insert in the *bst3-1* and A144 mutant. Insert specific primers and the information for the flanking region were obtained from the CLiP website for *bst3* (https://www.chlamylibrary.org/allMutants). Appendix I has the list of primers used for confirmations.

For the mutant A144 flanking region was confirmed using the protocol in from (Pollock et al., 2017). Briefly, genomic DNA from the mutant was extracted and restriction digested with *AleI, NaeI, PmlI* and *PvuII*. Digested DNA was run on a gel and cleaned up with phenol chloroform and concentrated with ethanol. This was followed by ligation of the DNA to the adapter as per Pollock (2017). Primary and nested PCR was then carried out using adapter specific and cassette specific primers and the product was commercially sequenced. Primers specific to the identified gene were then made and both sides of the cassette was confirmed using PCR and sequencing.

Nucleic acid preparation and gene expression analysis

For genomic DNA extraction, standard phenol chloroform method followed by ethanol precipitated. Plasmid preps were done using Qiagen mini prep kits. RNA was extracted using Trizol reagent protocol provided by Invitrogen. DNA was removed by adding DNAse as described in the New England Biolabs Inc. protocol. RNA was cleaned and purified using the Zymo RNA Clean & Concentrator. 1 µg RNA per sample was used as template for cDNA, which was made using ProtoScript® First Strand cDNA Synthesis Kit (NEB, Ipswich, MA) as per manufacturer's instructions. 100ng RNA per sample was used to conduct qRTPCR using the Luna® Universal One-Step RT-qPCR Kit (NEB) as per manufacturer's instructions using QuantStudio 6. Actin primers were used for semi quantitive RT-PCR and *CBLP* primers were used as a control in quantitative real-time PCR (qPCR). The primers used for the respective coding sequences are listed in Appendix I.

Bioinformatics analyses

The proteome of Chlamydomonas was downloaded from Phytozome (www.phytozome.net/) and was submitted to SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/: classification and secondary structure prediction system for membrane proteins) to select for membrane proteins. Putative location of the membrane proteins was obtained from TargetP1.1 server (http://www.cbs.dtu.dk/services/TargetP/) or PredAlgo (giavap-genomes.ibpc.fr/predalgo/).

A search in the PFam database (http://pfam.xfam.org/search/batch) for bicarbonate transporters yielded a list of protein families that had bicarbonate transporter members in different species. These families belong to manually curated Clans (or superfamilies) , and have been grouped on the basis of specific domains known to be associated with bicarbonate transport. From our list of 5000 membrane proteins, we identified Chlamydomonas genes that fall into the clans and families that are associated with bicarbonate transport.

Gene expression analysis

RNA was extracted using Trizol reagent following the method provided by Invitrogen (Waltham USA). 1 µg RNA per sample was used as template for cDNA, which was made using ProtoScript® First Strand cDNA Synthesis Kit (NEB) as per manufacturer's instructions. 100ng RNA per sample was used to conduct qRT-PCR using the Luna® Universal One-Step RT-qPCR Kit from NEB as per manufacturer's instructions using QuantStudio 6. Actin primers were used for semi quantitative RT-PCR and CBLP primers were used as a control in quantitative real-time PCR (qPCR). The primers used for the respective coding sequences are listed in Appendix I.

In silico modelling of bestrophin-like proteins

The peptide sequence of BST3 was obtained from phytozome v12.1. Homology modelling of BST3 was achieved using Swiss-model webserver using *Klebsiella pneumonia* Bestrophin (PDB: 4DW8) as a template. The BST1 homopentamer model was submitted to energy minimization with gromos 43B1 forcefield and the electrostatic potential was calculated using atom partial charge using Swiss-PDBviewer (V4.01). Result are visualized on the molecular surface. BST1 homopentamer structure quality was obtained using the QMEAN score (Benkert et al., 2008).

Phylogenetic tree

Amino acid sequences for BST1 (Cre16.g662600.t1.2), 2 (Cre16.g663400.t2.1) and 3 (Cre16.g663450.t1.2) were BLASTED against NCBI Genbank (Benson et al., 1993) and Phytozome v12.1 (Goodstein et al., 2012)and the top hits downloaded. Additionally, amino aCid sequences encoding Homo sapiens BEST1 (SJM31533.1) and Klebsiella pneumoniae bestrophin (pdb_4WD8_A) were downloaded from NCBI Genbank to be included as the outgroup in the phylogenetic analysis. A total of 63 initial sequences were aligned in Geneious 11.1.4 (Kearse et al., 2012)using a ClustalW (Thompson et al., 1994) algorithm with the amino aCid substitution matrix BLOSUM62 (Henikoff and Henikoff, 1992). Duplicate sequences from the two databases (NCBI and Phytozome) and sequences with a pairwise percentage positive identity (BLOSUM62) of less than 70% were removed. The final alignment included 30 sequences and was manually trimmed to remove variable length ends from the sequences. The phylogenetic analysis was completed in MEGA X (Kumar et al., 1994). The best Maximum Likelihood (ML) model for phylogenetic analysis of the alignment was calculated using the Model Selection

function in MEGA X. A ML tree was constructed using the LG substitution model (Le and Gascuel, 2008) with Gamma distribution (5 discrete categories) and 500 bootstrap replicates. Adobe Illustrator CC was used to prepare the tree for publication.

Linkage analysis

Genetic crosses and tetrad analyses were performed as described in described (Moroney et al., 1986; Harris et al., 2009). CC124 (mt^-) and A144 (mt^+) were cultured in nitrogen free media overnight and combined for mating for one hour. 0.3 mL aliquots were plated on nitrogen free TAP plates that have 3% agar and kept in the dark for zygote formation for a fortnight. Zygotes were then plated on 1.2% agar TAP plates and incubated for meiosis. Tetrad dissection and linkage analyses was carried out by determining if the Paro resistant progeny were sick on low CO_2.

CHAPTER 3
A BIOINFORMATICS SEARCH FOR NOVEL CI TRANSPORTERS

Introduction

A crucial component of the CCM is bicarbonate uptake and transport. Several genes contributing to Ci transport have been identified and characterized in Chlamydomonas, there may be other unidentified genes important to the CCM (Jungnick et al., 2014). Membrane transporters which have been well characterized are HLA3 (Im and Grossman, 2002) and LCI1 (Burow et al., 1996; Ohnishi et al., 2010) on the plasma membrane and Nar1.2 (Wang and Spalding, 2014; Yamano et al., 2015) on the chloroplast membrane. Soluble proteins like LCIB and LCIC are hypothesized preventing CO_2 leakage from the pyrenoid of the cell (Wang and Spalding, 2006; Duanmu et al., 2009; Yamano et al., 2010; Wang and Spalding, 2014). CIA8 is another putative transporter which when knocked out has a “sick on low” CO_2 mutant with reduced Ci affinity (Machingura et al., 2017). Uncharacterized proteins like CCP1/2 and LCI11 have also been identified by transcriptomic and proteomic studies but their exact function is yet to be discovered.

Inorganic carbon is generally accumulated as HCO_3^-. Since HCO_3^- is charged, it cannot cross membranes as easily as CO_2. Transport of HCO_3^- requires facilitation by way of channel or active transporter proteins. One of the general features of these known Ci transporters is that they are regulated at transcriptional level, with a marked upregulation under limiting CO_2 conditions. A second feature is that these transporters are regulated by CIA5/CCM1, a transcription factor responsible for the expression of many CCM genes in Chlamydomonas (Fukuzawa et al., 2001; Xiang et al., 2001; Fang et al., 2012).

Over the years a member of Ci transporters have been discovered and characterized using varied methods. For instance, LCI1 was identified by screening a cDNA library with probes synthesized from low CO_2 cells (Burow et al., 1996). The probes did not detect LCI1 mRNA in cells from high CO_2 conditions. Hence, the conclusion was that the clones were expressed LCI1 only in limiting Ci conditions. HLA3, the other plasma membrane Ci transporter is an ATP-binding cassette type of protein. The gene was identified by analysis of restriction fragment differential expression in clones exposed to high light and limiting CO_2 (Im and Grossman, 2002). A genome-wide analysis of low CO_2 inducible genes was conducted in which Nar1.2/ LCIA and LCIB were identified(Chen et al., 1997; Miura et al., 2004). CCP1/2 were originally purified from membrane fractions as polypeptides encoded by a LIP36 gene, upregulated on low CO_2 conditions (Spalding and Jeffrey, 1989). Recent work has localized these proteins to the mitochondria (Atkinson et al., 2016). However, their involvement in Ci transport has not been established because RNAi mutants showed a sick in low CO_2 phenotype conditions but did not show a difference in Ci affinity (Pollock et al., 2004). CIA8 was discovered from a large mutagenesis screen where the mutant for *Cia8* had a sick phenotype in low CO_2 conditions with a reduced Ci affinity at pH 9 (Machingura et al., 2017). An RNA-seq study conducted in 2012 (Fang et al., 2012) found several hundred genes in Chlamydomonas which were upregulated in low CO_2 as well as controlled by CIA5. These were grouped in clusters depending on their response to CO_2 and presence or absence of CIA5.

The availability of genomic data coupled to increasing power of bioinformatics have provided new opportunities to search for unknown components. In this study, we

performed bioinformatics analyses of Chlamydomonas genes encoding membrane transporters. Knocking out genes by mutagenesis and looking for impaired growth phenotype has only been successful for a small number of genes in Chlamydomonas. Chlamydomonas is predicted to have 486 membranes transporters which fall into various categories of transport (Merchant et al., 2007). While these putative components of the CCM need further investigation to verify their functional roles, it is also inevitable that there are more components of the transport system that have not yet been identified. A complete understanding of the CCM will require full characterization of the many unknown components. The main objective of this chapter is to identify genes that may be associated with inorganic carbon uptake and transport in Chlamydomonas, with focus on membrane proteins that provide bicarbonate passage.

Result

Categorizing Chlamydomonas proteins

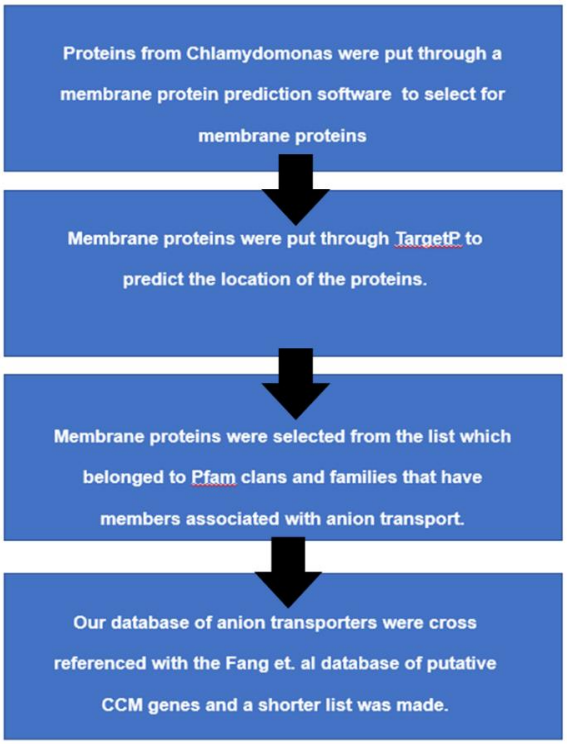
19000, predicted Chlamydomonas proteins were first run through a predictive search to identify membrane proteins. We used the online program SOSUI for this process. We selected only the membrane proteins because we were actively looking for Ci transporter like candidates. The membrane proteins were then passed through another predictive software called TargetP which predicts the putative location of these proteins.

Our next aim was to pick only those proteins that had domains that categorize them into transporter like families or superfamilies. The protein curation software Pfam which has 16712 families and 604 clans was used for this purpose. A quick keyword search with the words bicarbonate transport revealed every family in Pfam containing

members associated with transport. To broaden our search, we decided to include the clans with transporter like families as well. These clans were then cross referenced with our list of membrane transporters to pick up only the proteins that had transport domains. We obtained nearly 340 proteins in Chlamydomonas belonging to clans that had at least one member associated with bicarbonate transport.

Our knowledge of existing bicarbonate transporters reveals a few common features amongst them. HLA3, NAR1.2 and LCI1 are all upregulated under low CO_2 as well as controlled by CIA5 the transcription factor which controls most CCM genes. In order to further select Ci transporter like candidates we referred to the Fang transcriptomic list (Fang et al., 2012) and cross referenced our list of 340 genes to the genes in the clusters of the RNA seq data which were upregulated under low CO_2 and / or controlled by CIA5. Fig. 3.1A and B summarizes this process of obtaining 340 proteins and shows a Venn diagram intersection of the two datasets. We obtained 42 proteins from the list of putative membrane transporters which belong to the Fang et. al (2012) list as well. Table 3.1 has a list of the 42 candidates.

A



B

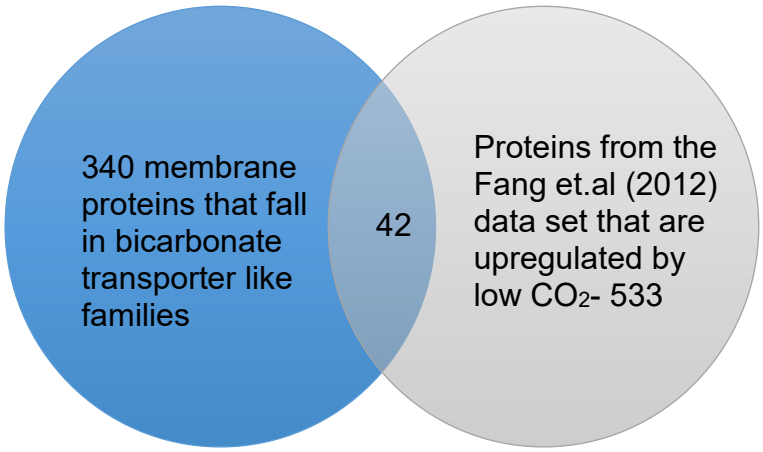


Fig. 3.1. A) A flowchart of the process used to get to the list of 42 putative transporter candidates which might play a role in the CCM. B)shows data sets obtained from the exhaustive search for Ci transporter like candidates that intersect with proteins from the Fang et. al dataset that are induced by low CO_2. 42 proteins fall into both datasets.

Table 3.1 The list of candidate genes which are upregulated in low CO_2 as well belong to transporter associated families in Pfam. Annotations have been obtained from Phytozome V12.1. The highlighted genes have been elaborated in Table 3.2 and fig. 3.2.

GENE ID	ANNOTATION
1. Cre06.g298750	Amino acid transporter
2. Cre17.g703800	Urea active transporter
3. Cre17.g723350	Sulphate ion transporter
4. Cre17.g714200	Potassium ion uptake transporter
5. Cre04.g214050	Sulphate transporter, MFS type
6. Cre16.g687450	$K^{(+)}$ Efflux antiporter 3
7. Cre05.g234645	Sodium/hydrogen exchanger 7 related
8. Cre10.g448100	Membrane transporter protein (Mem-trans)
9. Cre17.g733150	Response regulator receiver domain
10. Cre11.g467651	Adenylate and Guanylate cyclase catalytic domain
11. Cre16.g676800	Adenylate and Guanylate cyclase catalytic domain
12. Cre14.g613950	Lipid exporter ABCA1 and related proteins
13. Cre16.g655950	Transient receptor potential ion channel protein
14. Cre14.g618400	ATP-binding cassette transporter subfamily
15. Cre14.g627500	RecF/RecN/SMC N terminal domain
16. Cre14.g618600	ABC transporter, multidrug resistance associated protein
17. Cre16.g692300	ABC transporter transmembrane region
18. Cre16.g648700	Transporter, ABC superfamily
19. Cre16.g655450	ABC transporter G family member

(Table cont'd)

20. Cre17.g725150	Xenobiotic-transporting ATPase
21. Cre17.g723500	Plant PDR ABC transporter associated
22. Cre05.g234400	Pleiotropic drug resistance proteins
23. Cre02.g097800	ABC transporter
24. Cre01.g019850	FtsH-like ATPase/metalloprotease
25. Cre01.g007000	Transporter, ABC superfamily
26. Cre10.g443950	U-box domain
27. Cre04.g228650	Xenobiotic-transporting ATPase
28. Cre12.g540650	Plant PDR ABC transporter associated
29. Cre01.g031700	Methyltransferase FkbM domain
30. Cre10.g461750	C-5 cytosine-specific DNA methylase replication foci
31. Cre14.g612200	C-5 cytosine-specific DNA methylase
32. Cre17.g729400	Adenylate/guanylate cyclase
33. Cre17.g729350	Leucine-rich repeat-containing protein
34. Cre05.g237800	Leucine-rich repeat-containing protein
35. Cre02.g081800	Guanylate cyclase / Guanylyl cyclase
36. Cre04.g223350	Leucine-rich repeat-containing protein
37. Cre16.g662600	**Bestrophin associated gene- BST1**
38. Cre16.g663400	**Low CO_2 inducible gene 11 (LCI11)- BST2**
39. Cre16.g663450	**Low CO_2 inducible gene 11 (LCI11)- BST3**
40. Cre02.g097800	ABC transporter/ High light activated gene 3 (HLA3)

41. Cre03.g162800	Low CO_2 inducible gene 1 (LCI1)
42. Cre06.g309000	Anion transporter/ NAR1.2/LCIA
43. Cre07.g341925	BAND 3 Like

Confirming the upregulation of the selected genes

Some of the candidates of the list of 42 were already established as Ci transporters such as HLA3, NAR1.2, LCI1 etc. The function of LCI11(s) and the Bestrophin like genes will be described in a later chapter. The focus was on the 36 candidates which were novel. RNA was extracted from D66 and *cia5* cells which had been grown to log phase in acetate media, followed by either acclimation to high CO_2 or low CO_2. cDNA was then made from the RNA and semi quantitative qPCR was carried out with primers specific to 36 genes. Specific primers were made for each gene to confirm their upregulation in low CO_2 as well as control by CIA5. The PCR was run in cycles of 30, 35 and 39 to see a clear change in transcript abundance.

Fig 3.2 clearly shows the upregulation of 7 of the 36 tested genes in low CO_2 as well as control by CIA5. LCI1 has been used as a positive control which shows a clear upregulation in low CO_2 and little to no expression in high CO_2 and *cia5* mutant cells which is consistent with literature. Actin is used as a loading control which shows no change in expression under the condition used. The genes 3 and 21 resemble LCI1 with no expression in any of the conditions except for D66 in low CO_2. Genes 8,13 and 17 don't have a big difference in abundance between high and low CO_2 but have no expression in *cia5* which shows that they are controlled by CIA5. The list of interesting

genes with their gene IDs, putative locations from TargetP, annotations and families they belong to have been put together in Table 3.2.

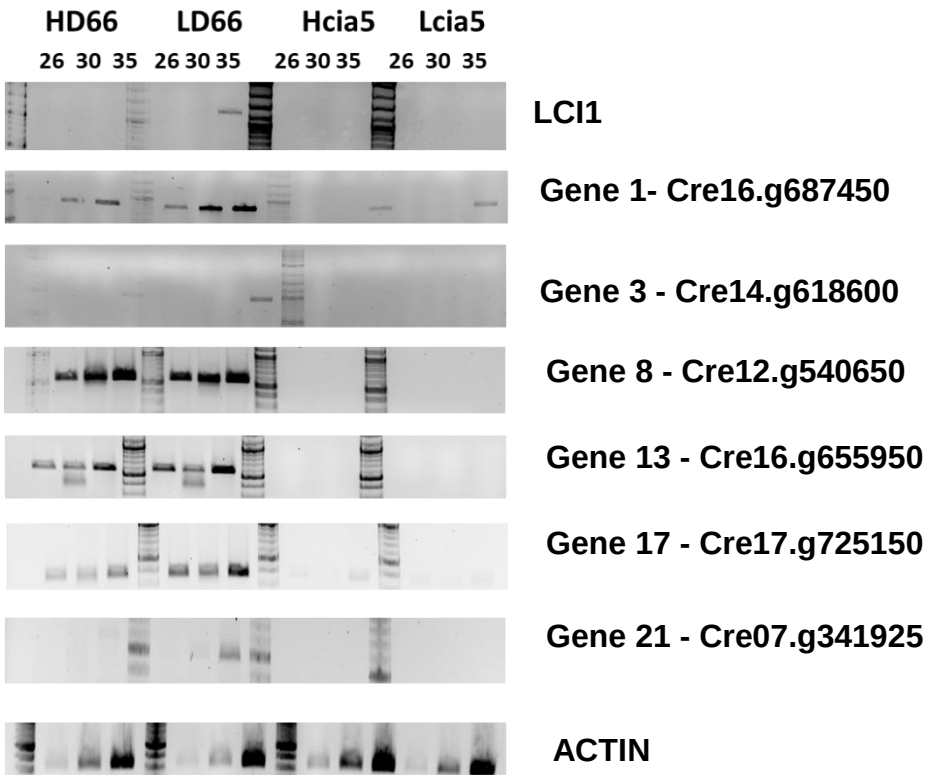


Fig. 3.2. shows the transcript abundance of the 7 genes which are upregulated in low CO_2, LCI1 was the positive control and actin was the loading control. Every gene is tested under high and low CO_2 using D66 cells as well as *cia5* cells. The transcript abundance is observed every 26, 30 and 35 cycles to be able to see a difference in expression. HD66- D66 cells grown in High CO_2, LD66- D66 cells grown in low CO_2, Hcia5- cia5 mutant cells grown in high CO_2, Lcia5- cia5 mutant cells grown in low CO_2.

Table 3.2 The seven genes which show transcript abundance in low CO_2 as well as low expression in high CO_2 with their respective locations from TargetP and PredAlgo. The gene IDs and annotations are taken from Phytozome.

	GENE ID	ANNOTATION	LOCATION
GENE 1	Cre16.g687450	Potassium proton antiporter	thylakoid
GENE 3	Cre14.g618600	ABC transporter, multidrug resistance protein	chloroplast
GENE 8	Cre12.g540650	Plant PDR ABC transporter associated	chloroplast
GENE 13	Cre16.g655950	Transient receptor potential ion channel protein	other
GENE 17	Cre17.g725150	Xenobiotic-transporting ATPase	other
GENE 21	Cre07.g341925	BAND 3 like	chloroplast

Discussion

Ci transporters are vital components of the CCM in green algae. Ci is brought into the cell in the form of bicarbonate since its an ion that will not enter and leave the cell as easily as a small uncharged molecule like CO_2.In Chlamydomonas there are three well characterized transporters which are LCI1, HLA3 and LCIA/NAR1.2. The former two are located on the plasma membrane and the latter is located on the chloroplast envelope. However recent data obtained from single mutants show clearly that all of these transporters have redundant functions. One such example is the LCIA knockout mutant which neither has a “sick on low“ CO_2 phenotype nor does it have any reduction in Ci affinity at pH 7.2 (Yamano et al., 2015). However, when HLA3 is also knocked out the double mutant for both transporters is both “sick on low” CO_2 as well has a marked difference in Ci affinity from the wild type (Yamano et al., 2015). Despite new data from transcriptomic and proteomic studies regarding the CCM in Chlamydomonas, our picture of the Chlamydomonas CCM is far from complete,

especially with respect to transporters. For instance, there is no known thylakoid transporter, which brings in bicarbonate for CAH3 in the lumen that can then release CO_2 to Rubisco to form PGA. It can also be hypothesized that there is more than one chloroplast envelope bicarbonate transporter in Chlamydomonas. This is inferred from a lack of phenotype or difference in Ci affinity in LCIA mutants.

Our method involved taking into account all proteins of Chlamydomonas and sorting them based on their predictive functions and locations. Predictive software was used to separate the membrane proteins from the soluble proteins and their putative location was obtained from TargetP/PredAlgo. Pfam was then used to categorize each membrane protein into families to have a better understanding of their function. In order to select for Ci transporter like candidates, only proteins belonging to families or clans with anion/ cation transporter associated members were selected. This includes families like ABC transporters, Band3 associated families, chloride channels etc. This analysis resulted in a list of hundreds of candidates which was then cross referenced with RNA-Seq data to pick for candidates which are induced by low CO_2 and/or controlled by CIA5. 42 proteins were selected which had members like HLA3, LCIA, LCI1 that are known Ci transporters in Chlamydomonas along with LCI11 and bestrophins which will be discussed in more detail in subsequent chapters.

In order to confirm that these candidates were indeed induced by low CO_2 and/or controlled by CIA5 semi quantitative RT-PCR was carried out. Six of the selected genes were upregulated on low CO_2 and had no expression in the *cia5* mutant. These genes belong to a variety of families such as a potassium antiporter, an ABC transporter, an ion transporter etc. It will be interesting to see what roles, if any, they play in the CCM

and if they directly transport Ci or are accessories in the process. Recent proteomic data already shows that gene 1 or the potassium antiporter in our list, which is also known as CPLD54 (Conserved in Land Plants protein) interacts with LCI11 and is localized to the thylakoid(Mackinder et al., 2017). This is interesting because CPLD54 could be a possible thylakoid transporter like candidate or it could be assisting LCI11 to bring in bicarbonate (see chapter 4 on bestrophins). Another interesting candidate could be gene 13 which belongs to a family of calcium channels and recent data shows a newly characterized calcium sensor protein which helps maintain the expression of several CCM transporters(Yamano et al., 2018). It is possible that gene 13 plays an indirect role in the CCM via calcium signaling.

Single mutants are available for some of these genes in the mutant library for Chlamydomonas. Alternately RNAi constructs can be made targeting these genes to better characterize their function in the CCM of Chlamydomonas. Preliminary screening of single mutants for gene 1 and gene 13 has shown no difference in phenotype or Ci affinity when compared to WT (Data not shown). This is consistent with the data obtained from single mutants for existing Ci transporters. Thus knocking down more than one gene or knocking down a known CCM transporter along with one of these genes may shed some more light on the mechanism of these genes and their contribution to the CCM.

CHAPTER 4
THREE THYLAKOID LOCALIZED BESTROPHIN-LIKE PROTEINS ARE ESSENTIAL FOR THE CO_2 CONCENTRATING MECHANISM OF *CHLAMYDOMONAS REINHARDTII.*

Introduction

The CCM of the unicellular green alga Chlamydomonas has several key properties. First, Chlamydomonas has a number of bicarbonate transporters that help increase the bicarbonate (HCO_3^-) concentration in the chloroplast stroma relative to the external HCO_3^- concentration. These transporters are located on the plasma membrane (LCI1 and HLA3) as well as the chloroplast envelope (NAR1.2/LCIA). Loss of any one of these transporters reduces the ability of the cell to accumulate HCO_3^- at high external pH (Wang and Spalding, 2014; Yamano et al., 2015; Machingura et al., 2017). Secondly, Rubisco is tightly packaged in a microcompartment of the chloroplast called the pyrenoid (Rawat et al., 1996; Borkhsenious et al., 1998; Mackinder et al., 2016). Finally, the carbonic anhydrase, CAH3 located in the chloroplast thylakoid lumen converts the HCO_3^- to CO_2 near the site of Rubisco. These CCM components, working together to increase the CO_2 concentration in the pyrenoid, enhance Rubisco carboxylase activity while decreasing photorespiration.

Chlamydomonas has many carbonic anhydrase (CA) genes encoding three α-type CAs, six β-type CAs and three γ-like CAs. Of these, CAH3, an α-type CA, has been shown to play an essential role in the CCM of Chlamydomonas (Moroney et al., 2011). CAH3 has also been shown to be located inside the thylakoid lumen (Karlsson et al., 1998). Loss of CAH3 results in cells that cannot grow on air levels of CO_2 even though these mutants tend to over-accumulate HCO_3^- (Karlsson et al., 1998). Chlamydomonas CCM

models propose that mutants missing CAH3 accumulate the HCO_3^- brought into the chloroplast by the transport proteins but cannot convert that HCO_3^- to CO_2, the actual substrate of Rubisco (Moroney and Ynalvez, 2007; Spalding, 2008). An interesting aspect of these CCM models is that they postulate that the pH gradient across the thylakoid membrane in the light helps drive the conversion of HCO_3^- to CO_2 (Raven, 1997). The pK_a of the interconversion of HCO_3^- and CO_2 is about 6.4 with the chloroplast stroma having a pH close to 8 in the light and the thylakoid lumen having a pH close to 5.5. So as HCO_3^- is brought from the stroma to the thylakoid lumen it goes from an environment favoring HCO_3^- to one favoring CO_2. Therefore, the acidification of the thylakoid lumen is important to the functioning of the CCM.

The CCM models also proposed the presence of a thylakoid bicarbonate transporter that brings in bicarbonate from the stroma to the lumen for dehydration by CAH3 (Moroney and Ynalvez, 2007; Spalding, 2008). In a recent interactome study, the CCM complex LCIB/LCIC is shown to be interacting with the bestrophin-like proteins encoded by Cre16.g662600 and Cre16.g66345 (Mackinder et al., 2017). These proteins were also shown to interact with each other, and another bestrophin-like protein encoded by Cre16.g663400 (Mackinder et al., 2017). All three genes were found to be upregulated on low CO_2 conditions in a transcriptomic study these genes belonged to a cluster of genes that had increased expression on low CO_2 and were controlled by CIA5 (Fang et al., 2012). Bestrophins are generally chloride transporters but have also been known to transport HCO_3^- anions (Qu and Hartzell, 2008). The interactome study also putatively localizes these bestrophin-like proteins to the thylakoid membrane which makes them

promising candidates to be the thylakoid HCO_3^- transporter in the CCM of Chlamydomonas.

In the present study we investigate the role of these three proteins using an RNAi approach to knockdown the expression of all three genes. This approach was feasible as the three genes are extremely similar at the DNA sequence level. Knockdown mutants with low expression of all three genes, grow poorly in limiting CO_2 conditions exhibit a poor affinity for external inorganic carbon and have a severely reduced ability to accumulate HCO_3^-. This study sheds light on the intracellular location and function of these bestrophin-like proteins in the CCM of Chlamydomonas.

Results

Chlamydomonas has three very similar Bestrophin-like proteins on the thylakoid membrane

BST1 (Cre16.g662600), *BST2* (Cre16.g663400) and *BST3* (Cre16.g663450) (collectively *BST1-3*) are paralogous bestrophin-like genes located within a 130 kbp region on the 16th chromosome of Chlamydomonas. Phylogenetic analyses revealed that bestrophin-like proteins are found in a diverse variety of photosynthetic organisms (Fig. 4.1) including vascular plants, non-vascular plants and diatoms. The amino acid sequences encoded by these genes were analyzed in TMHMM which predicted that BST1-3 are membrane proteins having four predicted transmembrane domains each. Further analysis using PredAlgo predicted that each BST protein had a chloroplast transit peptide and were likely to be chloroplast membrane proteins. BST1 was annotated as a bestrophin-like protein in Phytozome (ver. 12.1) and *BST2* and *BST3* were previously reported as *LCI11* by Fang et al. (Fang et al., 2012). An alignment between the three

Chlamydomonas bestrophin-like proteins showed that the proteins are >80% identical to one another (Fig 4.2). There are seven more genes annotated as encoding bestrophin-like proteins in the Chlamydomonas genome, but they share less than 50% identity to BST1-3. Sequence alignment of BST1-3 with human Bestrophin 1 (BEST1) showed low sequence identity between BEST1 and BST1-3 (21 - 23% Fig 4.2). The thylakoid localized VCCN1 protein of Arabidopsis has about a 30% sequence identity with BST1-3. To further explore the potential structure and function of BST1-3 we did homology modelling using Swiss-Model (Yang et al., 2014). Structural studies show that human and *Klebsiella pneumonia* bestrophins are pentameric, and modelling of BST1 in a pentameric assembly is of high confidence (Fig. 4.3A). The highest ranking template identified by Swiss-Model for BST1-3 was *K. pneumonia* bestrophin (kpBEST). BST1-3 contain non-polar residues along their selective pore that are conserved in proteins of the bestrophin family and are involved in anion transport (Fig. 4.3B; (Qu et al., 2006)). The entry pocket of BST1 has a predominantly negative electrostatic potential and the selective pore is neutral/positively charged, supporting the hypothesis that BST1-3 transport negatively charged ions (Fig. 4.3C, D) (Kane Dickson et al., 2014; Yang et al., 2014).

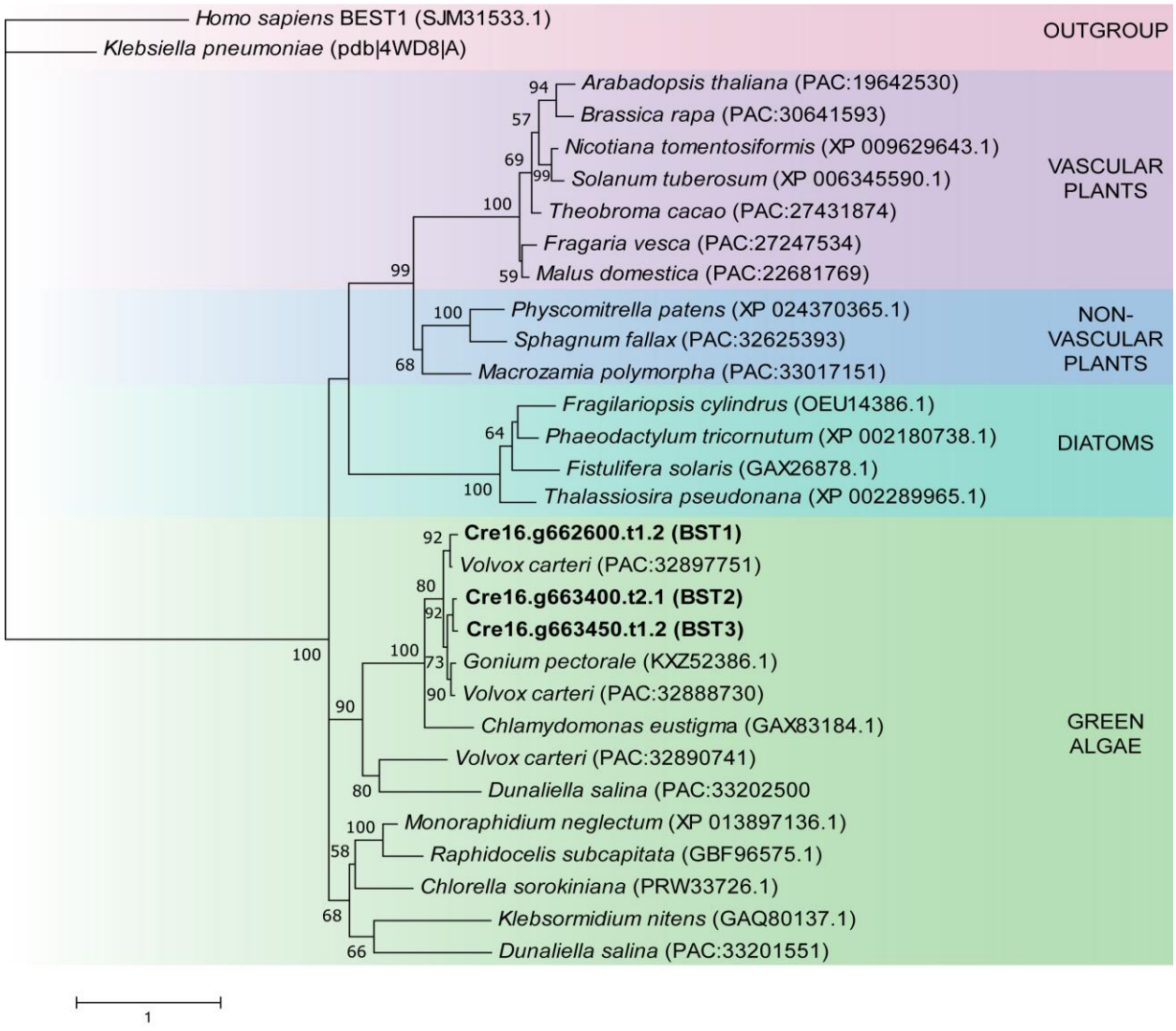


Figure 4.1. Phylogenetic analysis using Maximum Likelihood of Chlamydomonas bestrophin-like proteins BST1-3. The evolutionary history of Chlamydomonas bestrophin-like proteins BST1-3 was inferred by using the Maximum Likelihood method based on the Le Gascuel 2008 model with discrete Gamma distribution (5 categories) and 500 bootstrap replicates. Only branch support values of > 50 are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 4.2. Multiple sequence alignment (MSA) of BST1-3 vs. BEST1 and VCCN1 showing similarity between the three bestrophins, VCCN1 from Arabidopsis (Voltage gated chloride channel) and human bestrophin (BEST1). CLUSTALW was used for the multiple sequence alignment and TMHMM for predicting the TM domains in BSTs of Chlamydomonas. There is 80% similarity among BST1-3 and 30% between BST1-3 and VCCN1. Fully conserved residues are indicated by * (asterisk), conservation among groups of strongly similar properties are indicated by: (colon) and.(period) for weak properties.

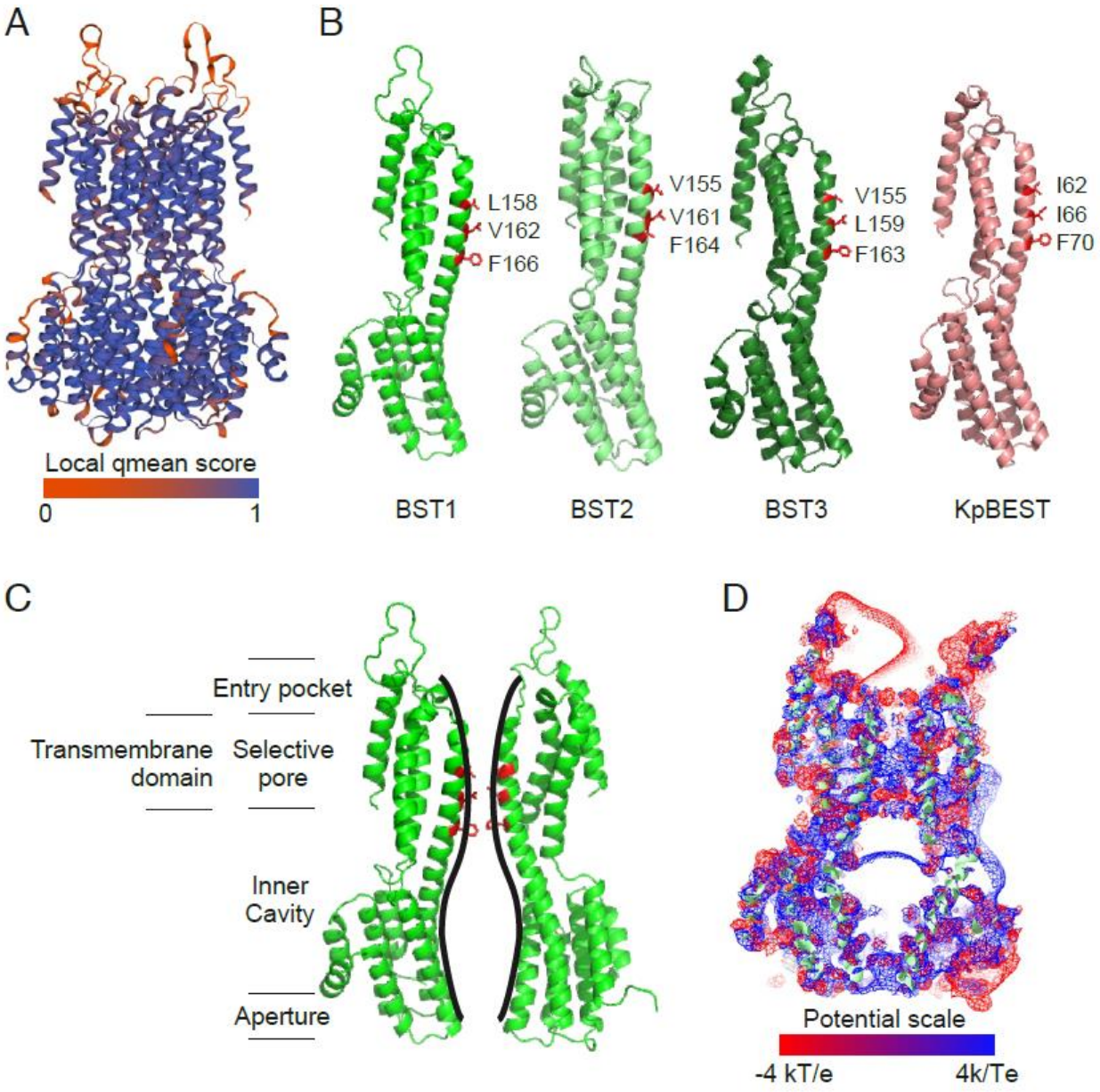


Figure 4.3. Structural models for BST 1-3. (A) Structural models for Chlamydomonas BST1-3 and *Klebsiella pneumonia* Bestrophin (kpbest). BST1-3 structural models were obtained using Swiss-model server with the *Klebsiella pneumonia* crystal structure (PDB: 4DW8) as template. The obtained structures and that of kpbest are displayed as monomers with conserved residues lining the selective pore highlighted in red. (B) Quaternary structure of BST1 pentamer model colored with the local QMEAN score - a structure quality assessment scoring function. (C) Outline of the channel cavity is drawn on BST1 homopentamer. (D) Calculated electrostatic potential is shown on BST1 model.

BST1-3 are thylakoid proteins up-regulated under low CO_2 growth conditions

Semi-quantitative RT-PCR (Fig. 4.4A) was performed using cDNA isolated from strains D66 and *cia5* grown under elevated CO_2 (5% CO_2 (v/v) in air) or low CO_2 (air with only 0.02% v/v CO_2) conditions. D66 is the wild-type strain for these studies and *cia5* is missing the CCM1 protein which is required for the induction of the CCM in Chlamydomonas (Moroney et al., 1989; Fukuzawa et al., 2001; Xiang et al., 2001). This work demonstrated that all three *BST* genes were up-regulated under low CO_2 growth conditions in D66 and that this up-regulation was not observed in *cia5.* In addition, the *cia5* mutant showed no expression for *BST1* and severely reduced expression of *BST3* under all conditions, a transcriptional pattern observed with other CCM genes. *BST2* transcript levels in *cia5* cells showed no noticeable induction in low CO_2 in contrast to D66 cells where *BST2* transcript levels increase in low CO_2 conditions. A time course study of the expression of these three genes was carried out using cDNA from high CO_2 grown cells which were transferred to low CO_2 levels for 2-12 h (Fig. 4.4B). All three genes had increased transcript levels within 2 h after the switch to low CO_2 and these levels of expression were maintained until at least 12 h after induction.

To better determine the localization of these three BST-like proteins in Chlamydomonas, fluorescent protein fusions were constructed linking Venus to the C-terminus of each BST protein. All three BST-like proteins localized to the thylakoid membranes of the chloroplast (Fig. 4.5A) and this localization visibly extended into the thylakoid tubules of the pyrenoid (shown for BST1; Fig. 4.5B). Thus, BST1, BST2 and BST3 are thylakoid localized anion transporters that are expressed coordinately with the expression of other Chlamydomonas CCM proteins.

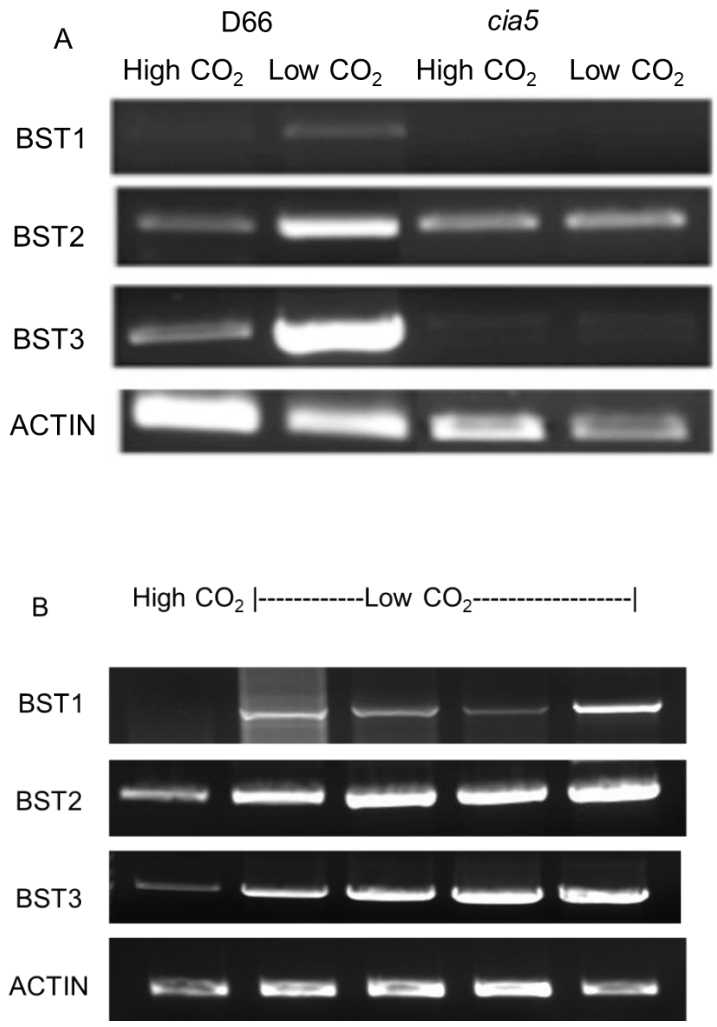


Figure 4.4. Transcript analysis of *BST1-3*. (A) Semi-quantitative RT-PCR showing *BST1-3* accumulation in low CO_2 (<0.04% CO_2 in air) vs. high CO_2 (5% (v/v) CO_2 in air) in D66 and *cia5* cells. B) Semi-quantitative time course showing the expression of *BST1-3* in cDNA obtained from high CO_2 (5% CO_2 (v/v) in air) and in cells switched to low CO_2 (<0.04% CO_2 in air) for the indicated times. Actin has been used as a loading control in both panels.

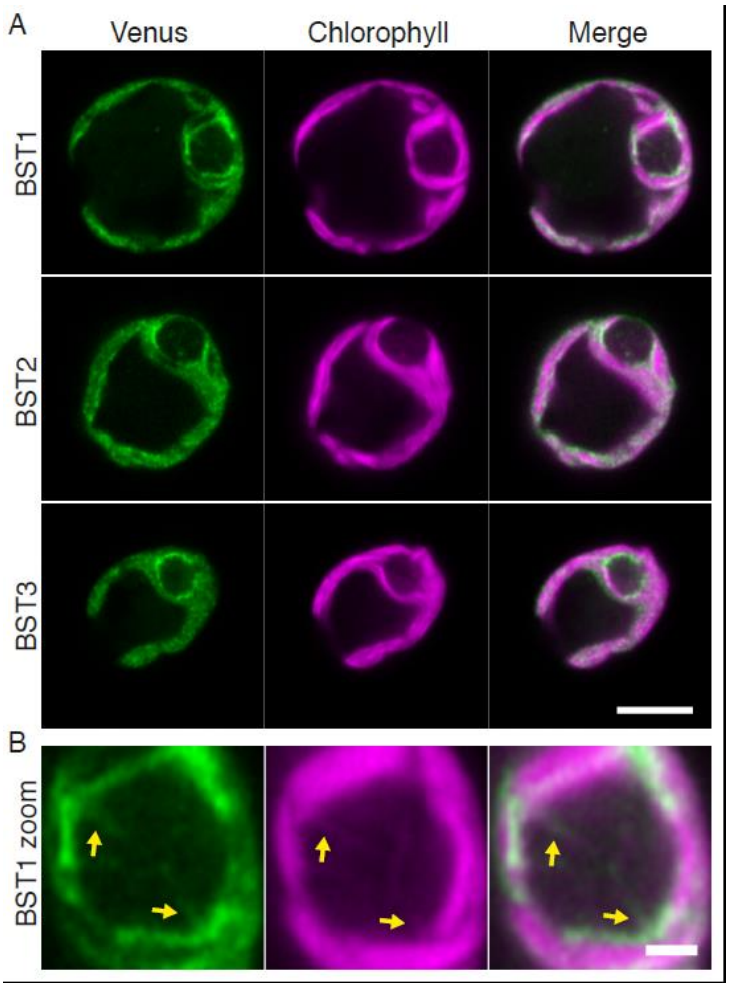
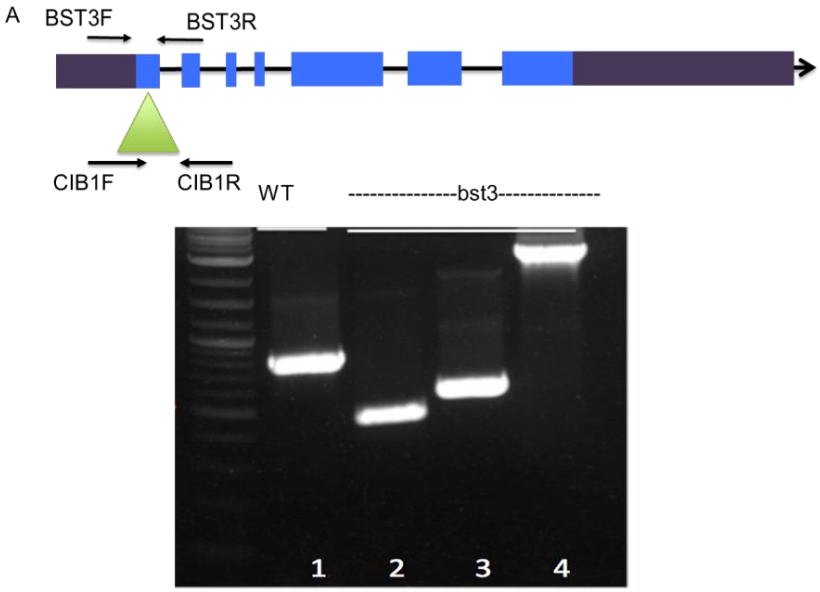


Figure 4.5. Localization of BST1-3. (A) Confocal microscopy of BST1-3 proteins fused with Venus (green). Chlorophyll autofluorescence is shown in magenta. Scale bar: 5 μm. (B) Zoom in image of BST1 pyrenoid shown in A. Yellow arrows highlight where BST1-Venus fluorescence is seen overlapping with chlorophyll fluorescence in the pyrenoid matrix. Scale bar: 1 μm.

Reduction of BST1-3 expression results in cells that grow slowly under low CO_2 conditions

A BST3 knockout (*bst*3) was obtained from the CLiP mutant collection with a paromomycin insert in the first exon of the *bst3* gene (Fig. 4.6A). In addition, the BST3 transcript was not detected in *bst3* (Fig. 4.6B). We observed no significant difference in growth or chlorophyll concentration for this strain as compared to wild-type cells under low (ambient CO_2) (Fig. 4.7AB). There also was no notable difference in Ci affinity

between WT and *bst3* (Fig. 4.7C). This supports the hypothesis that the function of the three BSTs might be redundant and that the expression of all three genes must be reduced to determine their physiological role(s). Therefore, to elucidate the function of *BST1-3* RNAi constructs complementary to regions of identity among *BST1-3* were designed and the D66 strain was transformed with these constructs. *bsti-1* and *bsti-2* (BST-RNAi lines 1 and 2) were isolated from 400 transformants and screened for a growth difference under very low CO_2, compared to D66. The growth of *bsti-1* on high, low and very low CO_2 was compared to D66, *pmp*1 and *cia3* (Fig. 4.8A). The strains *pmp1* (Wang and Spalding, 2006) and *cia3* are CCM mutants, with *pmp1* having a knockout of LCIB and *cia3* having a knockout of *CAH3*, the gene encoding the thylakoid lumen carbonic anhydrase (Karlsson et al., 1998). In very low CO_2, *bsti-1* showed severely reduced growth that was further exacerbated at high pH, resembling the growth of CCM mutants, *cia3* and *pmp1* (Fig. 4.8A). However, at high CO_2 the growth of *bsti-1* was comparable to wild-type, *cia3* and *pmp1*. Thus, the BSTs are required for wild-type like growth of Chlamydomonas under low CO_2 conditions. qRT-PCR was conducted using the cDNA of D66 and *bsti-1* and *bsti-2*. *bsti-1* and *bsti-2* showed nearly an 60-90% knockdown in the expression of *BST1*, *BST2* and *BST3* compared to D66 (Fig. 4.8B).



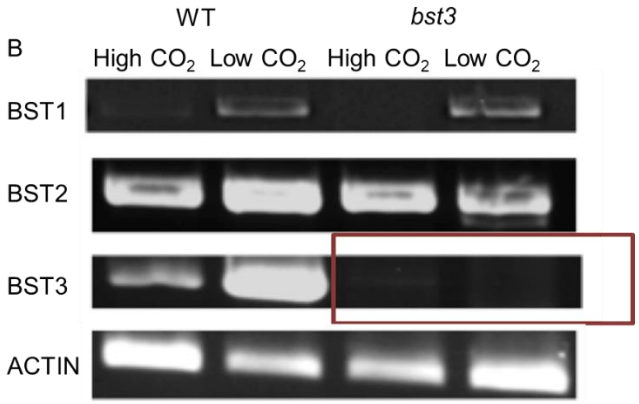


Figure 4.6. BST3 is knocked out in the CLiP mutant bst3. (A) The position of the insert in the BST3 gene and the PCR reactions used in the confirmation of the insert in *bst3*. The insert position was confirmed with primers specific for the insert and to the gene. Lane 1, BST3F and BST3R primers using CLIP-WT DNA as template; lane 2, CIB1F (insert) and BST3F primers using *bst3* DNA as template; lane 3, CIB1R (insert) and BST3R primers using bst3 DNA as template; lane 4, BST3F and BST3R primers using bst3 DNA as template. The size difference between lane 1 and 4 shows that there is an 1800bp cassette. B) RT-PCR shows that BST3 is knocked out in *bst3* but BST1 and BST2 are not affected. Actin is used as loading control.

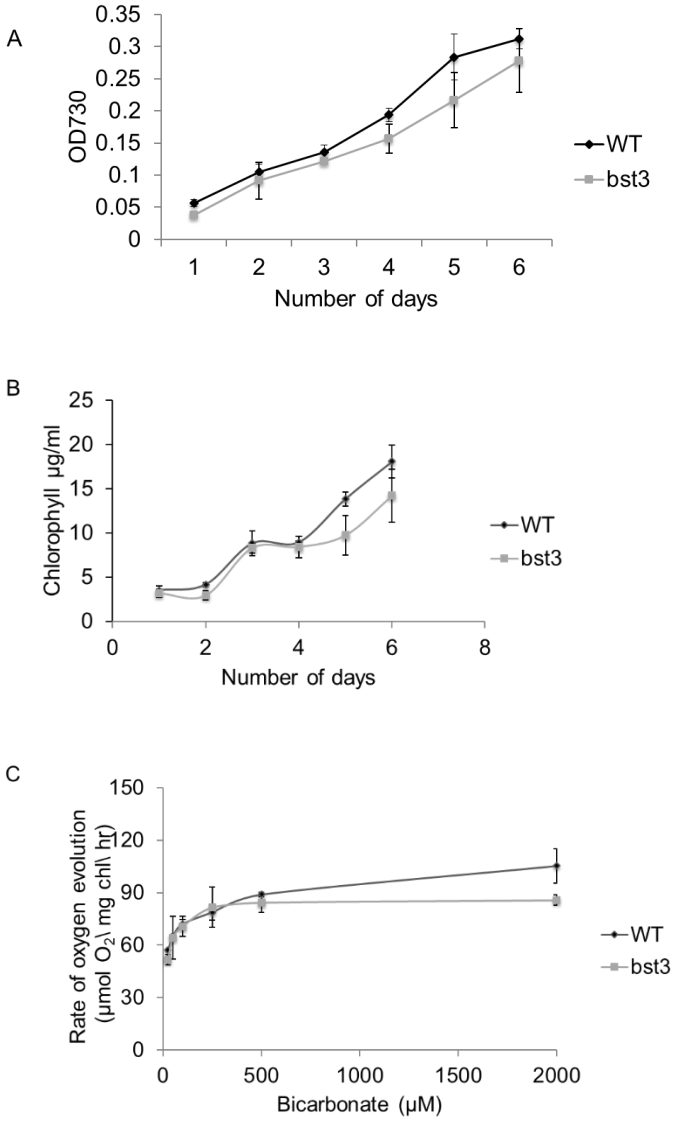


Figure 4.7. Growth and inorganic carbon affinity of bst3. (A) Growth of WT vs. *bst3* in pH 8.6. Growth was measured using OD730 and chlorophyll estimation at wavelength 645 and 663. Measurements were done at low CO_2 (<0.04% CO_2) for six days. Cells were grown in TAP for 48 hours before transferring them to MIN at an OD730 of 0.01. (B) Oxygen evolving activity was measured at pH 7.4 and the $K_{0.5}$(Ci) values (Ci concentration needed for half maximum oxygen evolution) were calculated from the O2 evolution versus Ci curves. Triplicate runs were made at each Ci concentration.

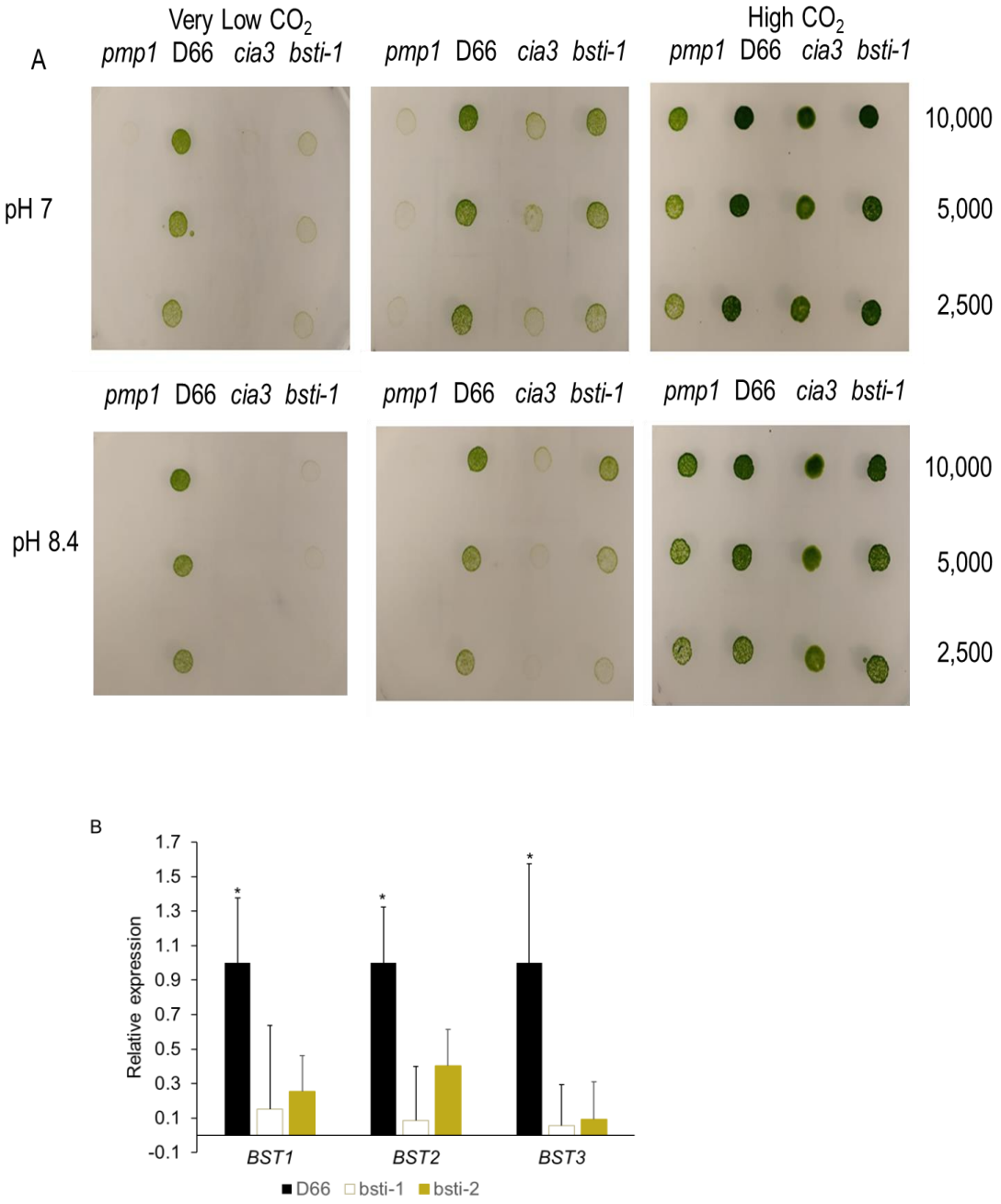


Figure 4.8. Growth of *bsti-1* and relative expression of *BST1-3* in *bsti-1*. (A) Spot tests showing growth of *pmp1*, D66, *cia3* and *bsti-1*. Cells were diluted to an OD_{730} of 0.1, 0.05 and 0.025 to compare growth in very low CO_2 (0.01% CO_2 (v/v) in air), low CO_2 (0.04% CO_2 (v/v) in air), high CO_2 (5% CO_2 (v/v) in air). Cells were grown for 2 days. (B) qRT-PCR shows that the expression of all three *BST* genes in *bsti-1* is reduced when compared to their expression levels seen in D66. D66 and *bsti-1* were acclimated to air levels of CO_2 for 12 hours before harvesting the RNA. All three *BST* genes show at least an 80% reduction in relative expression. $P < 0.05$ by student t-test.

Reduction of BST1-3 expression results in cells that have a reduced capacity to accumulate inorganic carbon

Two characteristics of algal cells with a CCM are their very high affinity for inorganic carbon (Ci) and having the ability to accumulate Ci to levels higher than can be obtained by diffusion. *bsti-1* and *bsti-2* acclimated to low CO_2 exhibited a three to tenfold lower affinity for Ci at pH 7.8 judged by their measured $K_{0.5}$(Ci) (Fig. 4.9). When grown at high CO_2, *bsti-1* still had a reduction in Ci affinity when compared to D66 grown at high CO_2 (Fig. 4.10). The maximal rates of photosynthesis for *bsti-1* was similar to D66 whether the cells were acclimated to low or high CO_2. This indicates that reducing the expression of all three *BST* genes caused a reduction in the cells' affinity for Ci. At pH 8.4 the $K_{0.5}$(Ci) for *bsti-1* is 95 μM, in sharp contrast to a low $K_{0.5}$(Ci) of 35 μM for D66 (Fig. 4.9A). At the higher pH of 8.4, the predominant Ci species in the medium would be bicarbonate, thus the increased affinity of the cells for Ci reflects their ability to actively take up and utilize bicarbonate. For *bsti-1* where the expression of all three BST genes is reduced, there is a reduced Ci affinity at both pH 7.8 and pH 8.4, in contrast to *bst3*, the mutant only missing *BST3* expression, which has no difference in Ci affinity with WT (Fig. 4.7C). Thus we can conclude that BST1-3 are important for the CCM of Chlamydomonas. Ci uptake activity was also measured in D66 and *bsti-1* to evaluate the importance of BST1-3 in accumulation and fixation of Ci. Low CO_2 acclimated *bsti-1* had significantly lower accumulation and fixation of ^{14}Ci compared to D66 at both pH 7.8 as well as pH 8.4 (Fig. 4.11 A-D). At both pH 7.8 and 8.4, *bsti-1* accumulated ^{14}Ci to only 20 to 25% of the levels observed in D66 cells. This difference was seen at both the earliest time point (15 s) up to two minutes in the light were the D66 cells had used up most of the added ^{14}Ci.

These results indicate that BST1-3 have an important role to play in the Ci uptake in low CO_2 conditions in Chlamydomonas.

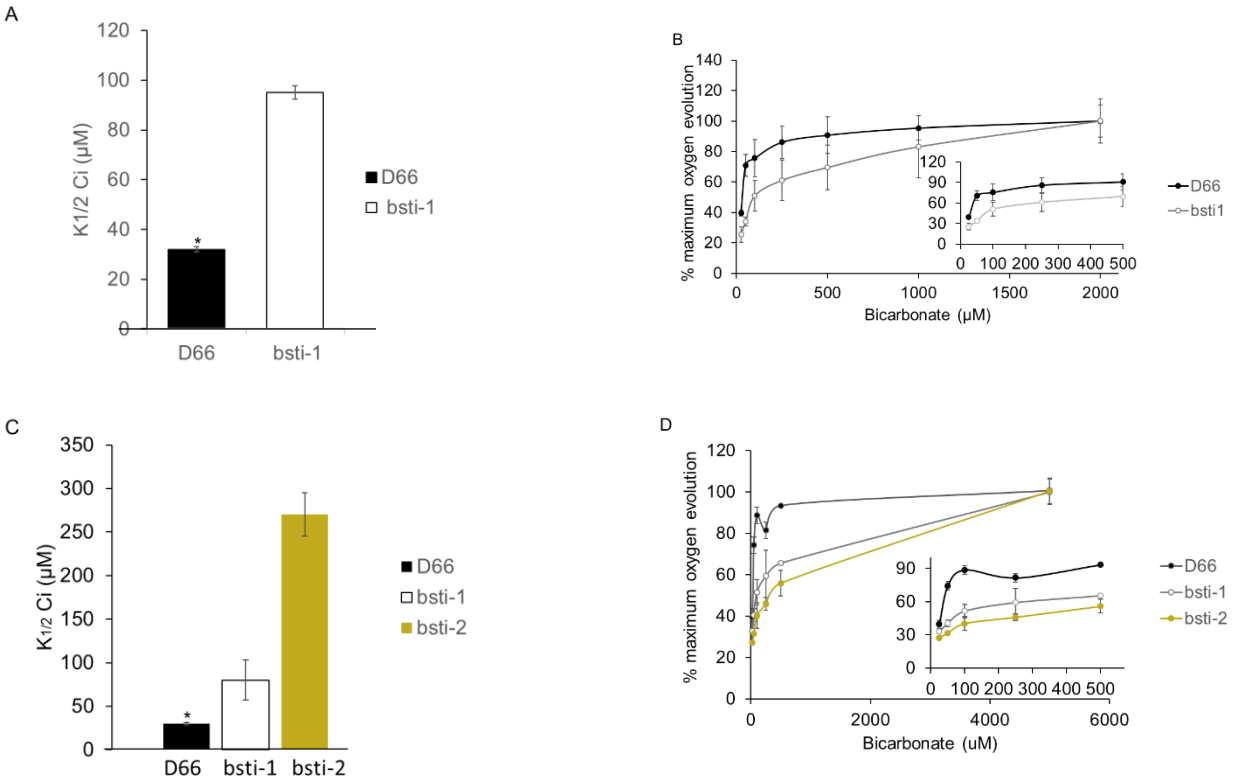


Figure 4.9. Photosynthetic oxygen evolution activity of *bst* RNAi lines and D66. Ci affinity was estimated for *bsti*-1 and D66 acclimated to low CO_2 (<0.04% CO2) for 12 h at pH 8.4 (A, B) and for *bsti-1, bsti-2* and D66 at pH 7.8 (C, D). Oxygen evolving activity was measured at the indicated pH and the $K_{0.5}$(Ci) values (Ci concentration needed for half maximum oxygen evolution) were calculated from the O_2 evolution versus Ci curves. Triplicate runs were made at each Ci concentration. * indicates that the differences in $K_{0.5}$(Ci) was significant ($P < 0.05$ by student t test). At pH 7.8, Vmax of D66 is 121μmol O_2 mg^{-1} Chl hr^{-1}, *bsti-1* is 105 μmol O_2 mg^{-1} Chl hr^{-1} and for *bsti-2* is 95 μmol O_2 mg^{-1} Chl hr^{-1}. At pH 8.4, Vmax of D66 is 124 μmol O_2 mg^{-1} Chl hr^{-1} and for *bsti-1* is 85.5 μmol O_2 mg^{-1} Chl hr^{-1}. The Vmax of all the strains have been set to 100% oxygen evolution activity. Error bars are all based on standard deviation.

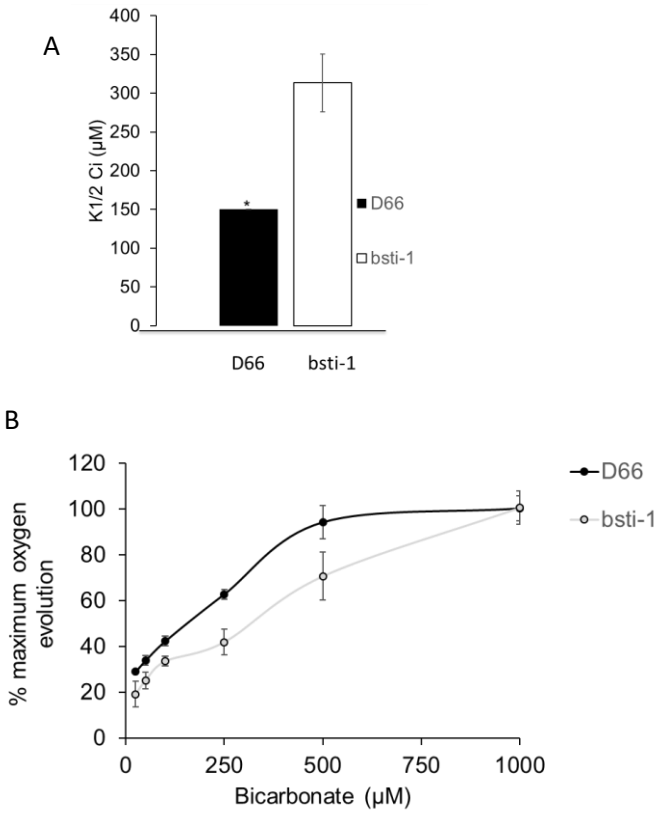


Figure 4.10. Photosynthetic oxygen evolution activity of *bsti-1* and D66 acclimated to high CO_2. Ci affinity was estimated for bsti-1 and D66 acclimated to high CO_2 (>5% CO_2) for 12 h at pH 7.8 (A, B). Oxygen evolving activity was measured and the K0.5(Ci) values (Ci concentration needed for half maximum oxygen evolution) were calculated from the O_2 evolution versus Ci curves. Triplicate runs were made at each Ci concentration. * indicates that the differences in $K_{0.5}$(Ci) was significant ($P < 0.05$ by student t test). V_{max} of D66 is 121µmol O_2 mg-1 Chl hr-1, bsti-1 is 120 µmol O_2 mg-1 Chl hr-1. The V_{max} of all the strains have been set to 100% oxygen evolution activity. Error bars are all based on standard deviation.

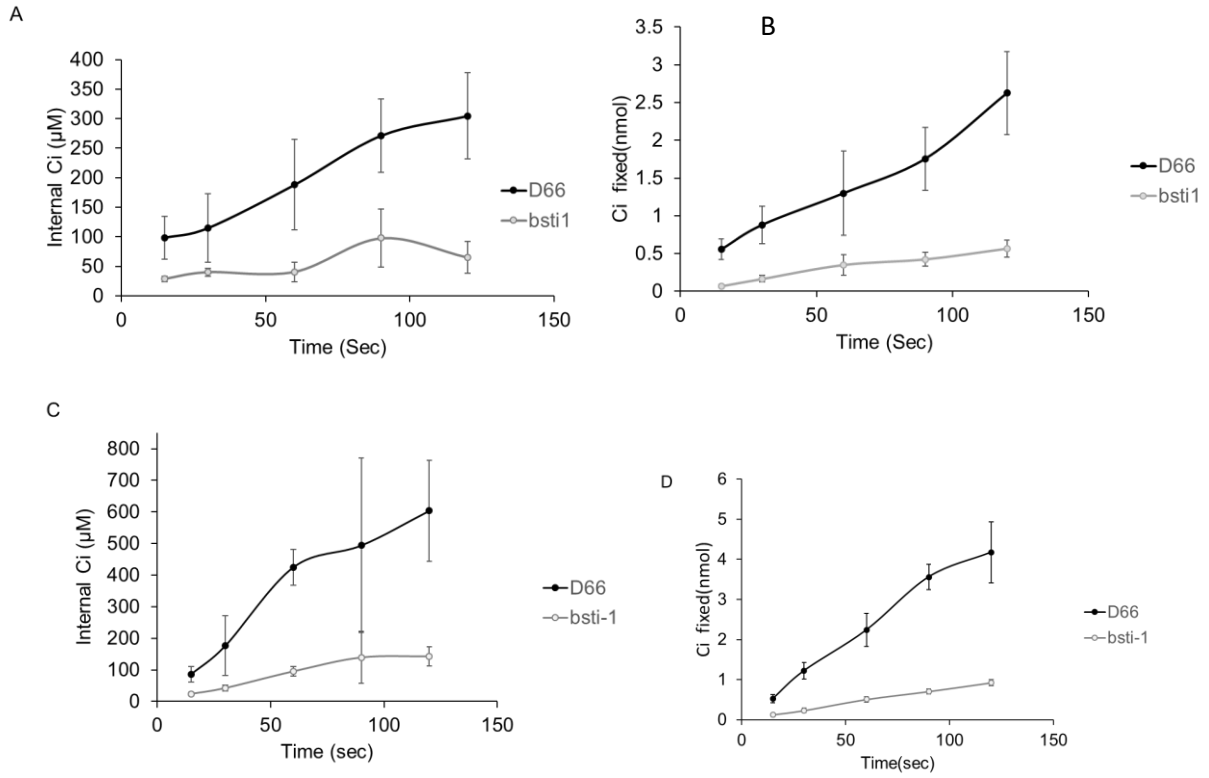


Figure 4.11. Inorganic carbon uptake of D66 and *bsti-1*. Ci uptake and Ci accumulation was estimated in D66 and *bsti-1* using the silicone oil uptake method (see materials and methods). Cells were grown in elevated CO_2 (5% CO_2 in air) and then acclimated to low CO_2 (<0.04% CO_2) for 12 h prior to the assays. Cells were harvested and depleted of endogenous Ci before running the assays. A time course of intracellular Ci accumulation (left) and CO_2 fixation (right) are shown for (A, B) pH 7.8 and (C, D) pH 8.4. Triplicate samples were run for each time point. The added $H^{14}CO_3^-$ concentration was 25 μM at pH 7.8 and 50 μM at pH 8.4.

Discussion

We present evidence here that BST1-3 are chloroplast thylakoid localized anion transporters that are important components of the Chlamydomonas CCM. Cells that have reduced *BST1-3* transcript levels fail to grow on low CO_2 have a lower affinity for inorganic carbon and have a reduced ability to accumulate added ^{14}Ci. The growth phenotype and inorganic uptake of *bsti-1* is similar to the *pmp1* mutant strain that has a knock-out of LCIB. A key aspect of current Chlamydomonas CCM models is that accumulated HCO_3^-

is converted to CO_2 by CAH3, a carbonic anhydrase located in the thylakoid lumen. This feature of algal CCMs may extend to other algal types, notably diatoms where Kikutani et al. (Kikutani et al., 2016) recently discovered a θ-type carbonic anhydrase within the thylakoid of *Phaeodactylum tricornutum* that was required for CCM function. These CCM models predict that a thylakoid HCO_3^- transporter is required to deliver HCO_3^- from the chloroplast stroma to the thylakoid lumen. We propose that BST1-3 are the transporters that bring HCO_3^- to CAH3 inside the thylakoid in Chlamydomonas.

Members of the human bestrophin family have been shown to transport HCO_3^- and Cl^- ions (Qu and Hartzell, 2008). The homology modeling presented here supports the function of BST1-3 as negative ion transporters, with BST1-3 having predicted structural similarities and conserved transport residues to chicken and bacterial bestrophins (Fig. 4.2).

The expression of all the CCM transporters discovered previously is induced by limiting CO_2 conditions and their expression is controlled by the transcription factor CIA5/CCM1 (Moroney et al., 1989; Fukuzawa et al., 2001; Xiang et al., 2001). We have observed that all three *BST* genes are induced when Chlamydomonas is grown under low CO_2 conditions and that this induction is absent in the *cia5* mutant. In addition, the expression of LCIB and LCIC, possible θ–carbonic anhydrases (Jin et al., 2016; Kikutani et al., 2016) essential to the CCM (Wang and Spalding, 2014) that interact with BST1-3 (Mackinder et al., 2017) . have the same expression pattern. Thus, the expression of the *BST1-3* genes is consistent with these proteins playing a role in the uptake and accumulation of inorganic carbon when Chlamydomonas is exposed to low CO_2 conditions.

In Chlamydomonas there seems to be a built in redundancy of Ci transporter functions. For example, both LCI1 and HLA3 are present on the plasma membrane and loss of only one of the proteins fails to cause an extreme growth phenotype at low CO_2 (Yamano et al., 2015). However, when more than one transporter is knocked down, a significant change in Ci uptake and growth is observed(Yamano et al., 2015; Machingura et al., 2017). BST1-3 also appear to have redundant or overlapping functions. This is demonstrated in this study as knocking out BST3 by itself did not cause a drastic change in growth or reduction in Ci affinity (fig. 4.7). However, when the expression of all three genes is decreased in *bsti-1,* cells could not grow at low CO_2 and inorganic carbon uptake was severely compromised (fig. 4.11). The strains *bsti-1 and bsti-2* have a greater than severe reduction in expression of *BST1-3* and show severe growth phenotypes and lowered affinity for inorganic carbon. This redundancy likely explains why these *BST1-3* genes were not identified in earlier mutant screens as these screens typically knock out only one gene at a time.

Three key components of algal CCM models are 1) bicarbonate transporters are required for HCO_3^- accumulation, 2) Rubisco must be packaged in a very specific location and 3) a carbonic anhydrase must be localized very close to the Rubisco converting accumulated HCO_3^- to CO_2. The CO_2 generated by this carbonic anhydrase close to Rubisco locally increases the CO_2 concentration, which increases its carboxylase activity and decreases its oxygenase activity. Fig.4.12 shows a refined model for the Chlamydomonas CCM which now includes our proposed function for BST1-3. In this model HLA3 and LCI1 transport HCO_3^- across the plasma membrane bringing HCO_3^- into the cell (Moroney and Ynalvez, 2007; Spalding, 2008). At the chloroplast envelope

NAR1.2 (LCIA) transports HCO_3^- into the chloroplast stroma. Then BST1-3 on the thylakoid brings HCO_3^- into the thylakoid lumen where CAH3 in the pyrenoidal thylakoid tubules converts HCO_3^- to CO_2 to be fixed by Rubisco in the pyrenoid. With the discovery of BST1-3, CCM researchers have now identified HCO_3^- transporters at each of the three membranes that HCO_3^- must cross to reach CAH3, namely the plasma membrane, the chloroplast envelope and the thylakoid membrane.

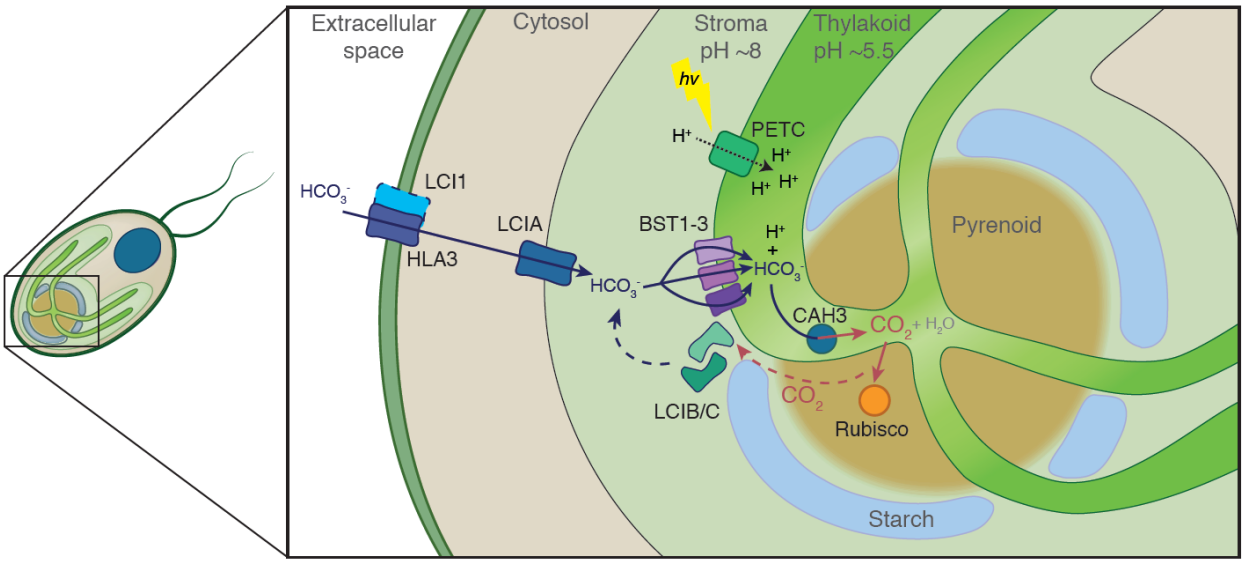


Figure 4.12. A tentative model showing the proposed physiological role of BST1-3 in the CCM of Chlamydomonas. Known transporters (LCI1, HLA3 and LCIA) are indicated on the plasma membrane and chloroplast respectively. Solid line arrows indicate the movement of HCO_3^- into the thylakoid by BST1-3. Dashed lines indicate the proposed leakage reducing pathway that involves recycling CO_2 by LCIB/C back to HCO_3^-. The dotted line represents the light driven establishment of a proton gradient across the thylakoid membrane by PSII and the cytochrome b6f complex of the Photosynthetic Electron Transport Chain (PETC).

However, many CCM models also propose that the algal cell must be able to recapture CO_2 that is generated by the CCM. This need to recapture inorganic carbon was first modeled in cyanobacteria (Kaplan and Reinhold, 1999), and is likely a general property of algal CCMs (Duanmu et al., 2009; Yamano et al., 2010; Wang and Spalding, 2014). A second CCM role of BST1-3 may be in inorganic carbon recapture (Fig. 4.12). As CO_2 is generated in the pyrenoid, any CO_2 that is not fixed by Rubisco has the potential to simply diffuse out of the cell. The LCIB/C complex is thought to help in this recapture of CO_2 (Duanmu et al., 2009). LCIB/C has been shown to coat or surround the pyrenoid, particularly when cells are grown under very low CO_2 conditions (Yamano et al., 2010). LCIB/C that has been proposed to function in low CO_2 conditions as a CO_2 recapture system in a directional manner driving CO_2 to HCO_3^- or to act as a tightly regulated carbonic anhydrase (Mackinder, 2018). This is particularly interesting because LCIB/C is believed to be interacting with BST1 and BST3 according to Mackinder et al. (Mackinder et al., 2017). Our model adds BST1-3 to this hypothesized recapture system (Fig. 3.12). Our localization work shows that BST1, BST2 and BST3 are all found on thylakoid membranes throughout the chloroplast. This would increase the surface area for the re-uptake of HCO_3^- in the stroma. As such, we propose that the recapture of inorganic carbon is a two-step process. In the first step, the leaked CO_2 from the pyrenoid is converted to HCO_3^- by LCIB/C at the pyrenoid surface. In the second step, BST1-3 brings the HCO_3^- back into the thylakoid creating an overall cyclic recapture mechanism. Loss of either BST1-3 or LCIB/C is predicted to reduce inorganic carbon accumulation as inorganic carbon leakage would be predicted to increase. This prediction agrees with the

experimental finding that loss of LCIB/C or reduction of BST1-3 results in cells that cannot accumulate inorganic carbon to normal levels.

The discovery of CCM components on the thylakoid (BST1-3) and inside the thylakoid lumen (CAH3) also indicates how light energy may be used to energize the CCM. The pK_a of the HCO_3^- to CO_2 interconversion is about 6.4. So the pH of the chloroplast stroma, thought to be near 8.0 is well above the pK_a while the pH of the thylakoid lumen is thought to be close to 5.5, below the pK_a. So when HCO_3^- moves from the chloroplast stroma to the thylakoid lumen it moves from an environment that favors HCO_3^- to one that favors CO_2. This effectively allows the algal cells to increase the CO_2 concentration to levels higher than could be obtained by the action of carbonic anhydrase alone. Thus, a trans-thylakoid pH gradient is necessary for this proposed "CO_2 pump" and this pH gradient set up by the photosystems and requires light (Raven, 1997; Moroney and Ynalvez, 2007; Spalding, 2008). To date, all experimental data available indicates that light and the activity of the photosystems is required for the Chlamydomonas CCM to function. In fact, some of the earliest work in the field indicated that electron transport inhibitors and mutations that disrupt electron transport also inhibited the Chlamydomonas CCM (Badger et al., 1980; Spalding et al., 1983). One potential problem with this CO_2 pump model is that it would partially reduce the proton motive force across the thylakoid membrane, thus reduCing ATP biosynthesis. However, it should be pointed out that only one H^+ would be consumed per CO_2 generated which is the equivalent of less than 1/3 of an ATP per CO_2 generated. This cost is far less than the two additional ATP required for C_4 photosynthesis and C_4 photosynthesis has been shown to be energetically competitive with C_3 photosynthesis once the costs of photorespiration are considered

(Moroney et al., 2013). This proposed role of photosynthetic electron transport in the CCM may not be limited to green algae. The recent discovery of the thylakoid luminal carbonic anhydrase in the diatom *Phaeodactylum tricornutum* supports the hypothesis that a light activated CO_2 pump might be operating in a wide diversity of algae (Kikutani et al., 2016).

In conclusion, BST1-3 are bestrophin-like, thylakoid localized membrane proteins which are synthesized in coordination with other CCM components and their predicted structures fit well with other anion transporters. As such, they are excellent candidates to be the HCO_3^- transporters that not only bring HCO_3^- into the thylakoid lumen for CO_2 generation but may also play a role in inorganic carbon recapture as well. The LCIB/C and BST1-3 recapture system may be engineered to be expressed in higher plants to genetically manipulate C3 plants to target the improvement of photosynthesis.

CHAPTER 5
CHARACTERIZATION OF ELONGATION FACTOR-3 IN CHLAMYDOMONAS

Introduction

Chlamydomonas is a model organism for many photosynthetic processes including the CCM. The reasons are it has a haploid genome, can grow autotrophically as well as heterotrophically and a sequenced genome- mitochondria, nuclear and chloroplast (Merchant et al., 2007; Harris et al., 2009). Proteomic and transcriptomic studies have predicted several new potential CCM candidates based on their expression in limiting CO_2 or control by vital transcription factors (Fang et al., 2012; Mackinder et al., 2017). However, the availability of mutants for these new candidates is vital to characterize them.

Forward genetics screen has been used successfully to identify mutants for vital CCM genes. This includes the mutant for CAH3, the thylakoid lumen CA (Spalding et al., 1983; Karlsson et al., 1998) as well as LCIB, the theta CA in the chloroplast stroma (Wang and Spalding, 2006). Even the mutant for CIA5, the master regulator of the CCM was identified via forward genetics screen (Moroney et al., 1989; Fukuzawa et al., 2001). However, there is a lack of mutant availability for several carbonic anhydrases and inorganic carbon transporters. For instance, there is no mutant available for LCI1, a plasma membrane transporter, which is known to interact with HLA3, another plasma membrane transporter (Mackinder et al., 2017). The contribution of LCI1 to the CCM can be better characterized with the help of a LCI1 knock out mutant. Similarly, the lack of a phenotype in a NAR1.2 mutant, the chloroplast transporter, supports the hypothesis that there may be more than one transporter on the chloroplast envelope, which is yet to be discovered. CAH4/5 are also intriguing carbonic anhydrases in Chlamydomonas

which are induced by limiting CO_2 but lack of a mutant has prevented further characterization of their role in the metabolism of Chlamydomonas. A forward genetics screen can be a useful tool in identifying essential components of the CCM as well as characterize putative components of the CCM.

The basic features of a forward genetics screen involves insertion of foreign DNA in the algae followed by screening of transformants for different levels of growth via a high vs. low CO_2 (Shimogawara et al., 1998). Mutants were selected based on paromomycin resistance. The paromomyCin cassette (ORF of *AphVIII* cassette) from the plasmid pSL18 was flanked by a dual promoter (*Hsp70* and *RbcsII*) on the 5' end and a RbcsII terminator on the 3' end. The linearized cassette was transformed into the wild type (D66) and antibiotic resistant colonies were selected. Colonies were screened on their growth in low CO_2 compared to wild type. Based on the sick on low CO_2 phenotype, mutants are selected and screened for the presence of the insertion. The general scheme of the process is shown in Fig. 5.1. Due to an absence of homologous recombination in Chlamydomonas the insertion of cassette is random. Since a sequenced genome database is available(Merchant et al., 2007) if the flanking region from the insert can be identified, the disrupted gene can be identified too. There has been variable degree of success localizing random DNA inserts using TAIL PCR, RESDA PCR or 3'RACE. Our lab developed an adapter mediated PCR method where genomic DNA from high CO_2 requiring mutants were used to identify the genes where the paromomycin insert was present, with a high degree of success(Pollock et al., 2017).

This chapter will summarize the basic steps that were undertaken to carry out a screen, outline the method to find the location of random inserts in Chlamydomonas as well as brief characterization of a sick on low CO_2 mutant.

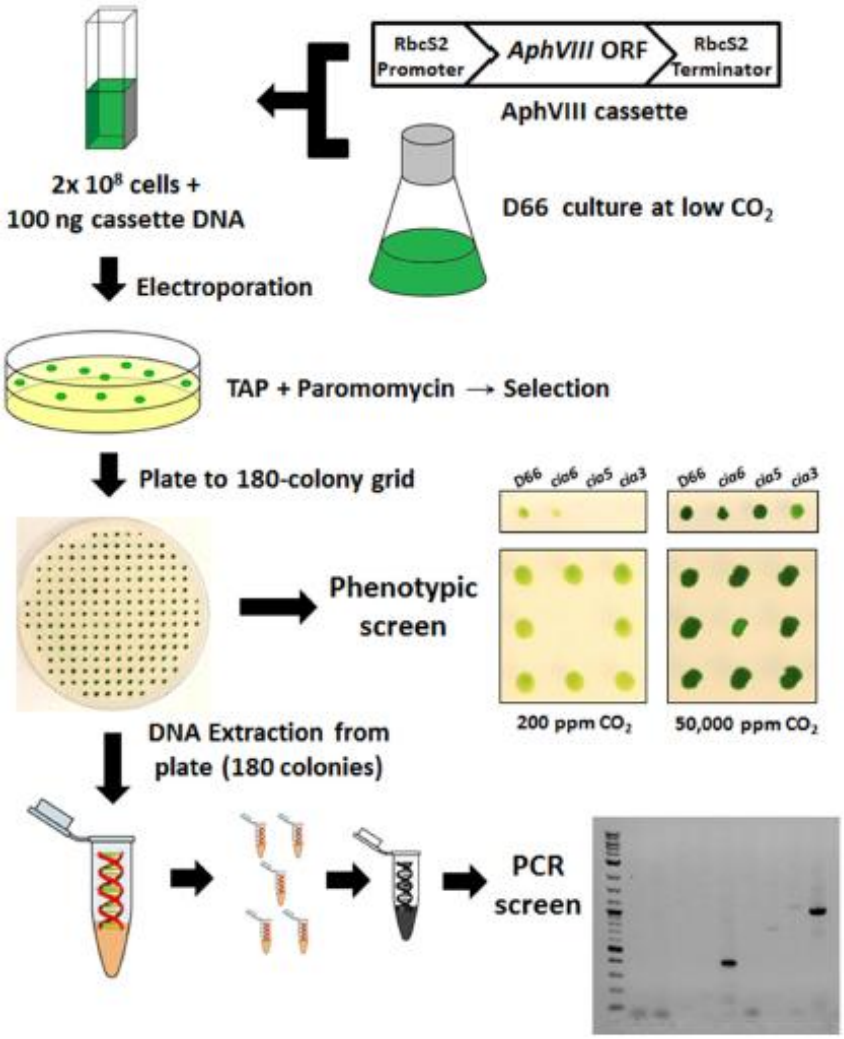


Figure 5.1. Schematic diagram of the mutagenesis screen. Cells in the log phase were transformed with the paromomyCin resistant cassette. Paromomycin was used to select for transformants. Colonies with a growth defect at low CO_2 were selected for adapter PCR in order to find the position of the cassette.

Results

Adapter PCR was used to find the location of the insert in colonies showing poor growth in low CO_2

Nearly 30,000 paromomyCin resistant mutants were generated from our large scale mutagenesis screen. 300 of those colonies had a visible sick on low CO_2 phenotype compared to the wild type (D66). It was important to know the identity of the genes in which the insert was present in these colonies. Genomic DNA was extracted from the mutant, followed by restriction digestion and blunt ligation of the adapter to the insert. Nested PCR with primers specific to the *AphVIII* cassette and adapter was carried out and the PCR product is sequenced. The sequence obtained was screened for homology against the Chlamydomonas genome in Phytozome database to identify the gene with the insertion. The scheme was briefly outlined in Fig. 5.2. One such mutant, A144 has an insertion in a gene annotated as an elongation factor- 3 and ATP binding cassette. The characterization of the A144 mutant will be discussed in detail in the following sections.

A144 has a growth defect compared to D66

A144 was grown in low, ambient and high CO_2 along with *cia3* (mutant for CAH3), *cia5* (mutant for CIA5), *cia6* (a mutant with a disrupted pyrenoid) and D66 (wild type) as shown in Fig. 5.3. D66 had normal levels of growth in all levels of CO_2 and *cia3, cia5* and *cia6* struggled to grow in limited CO_2. A144 is weaker in both low and ambient CO_2 compared to D66 but does not quite recover its phenotype in high CO_2 like other CCM mutants. This led us to consider that the function of the EF3 gene was for non CCM activities in the cell.

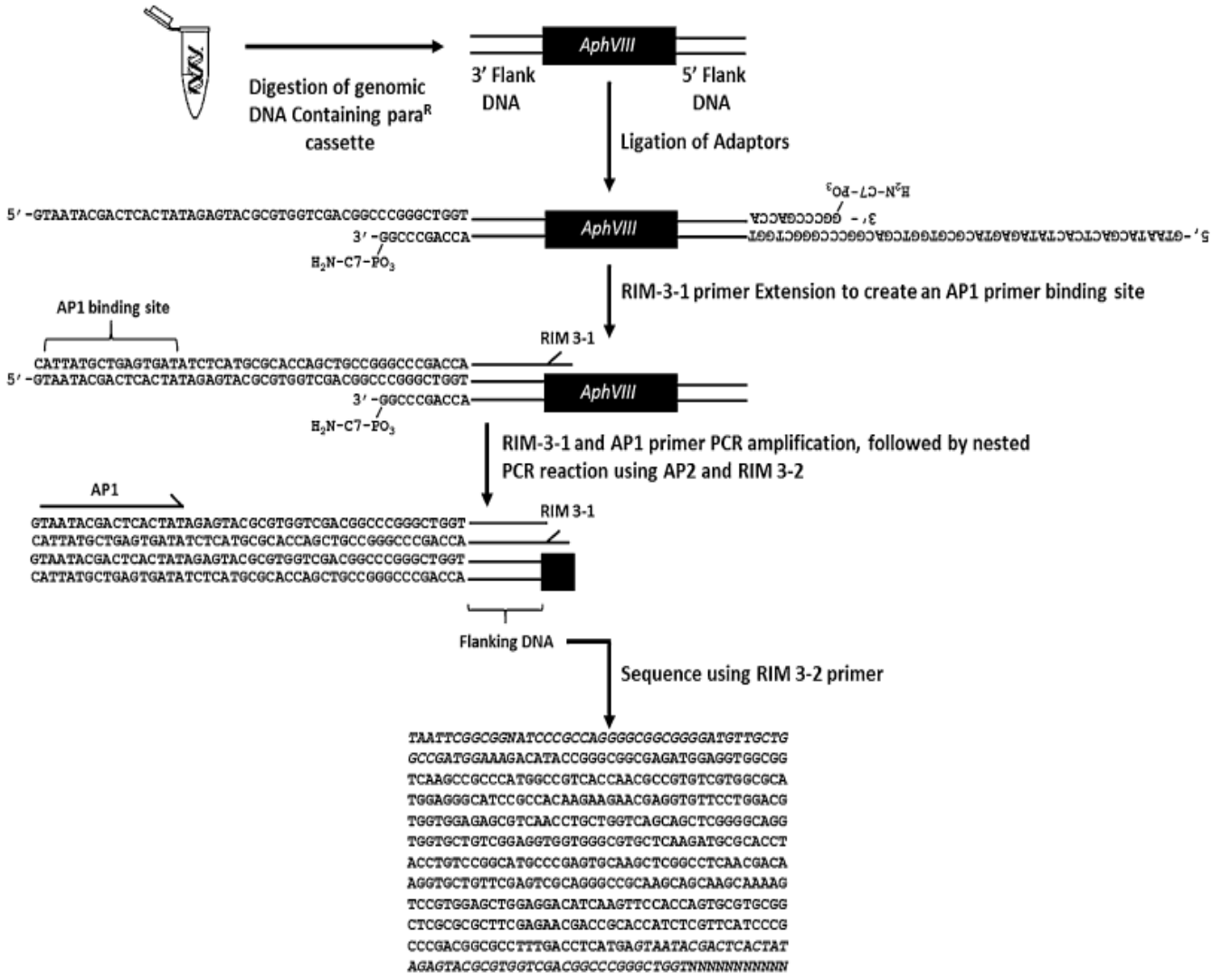


Figure 5.2. Schematic of the adapter PCR modified in our lab. Genomic DNA from the mutant is restriction digested and ligated to the adapter. Primers specific to the cassette and adapter are then used to amplify this DNA with nested PCR. The flanking regions are then sequenced from the PCR product to identify the gene where the cassette has been inserted.

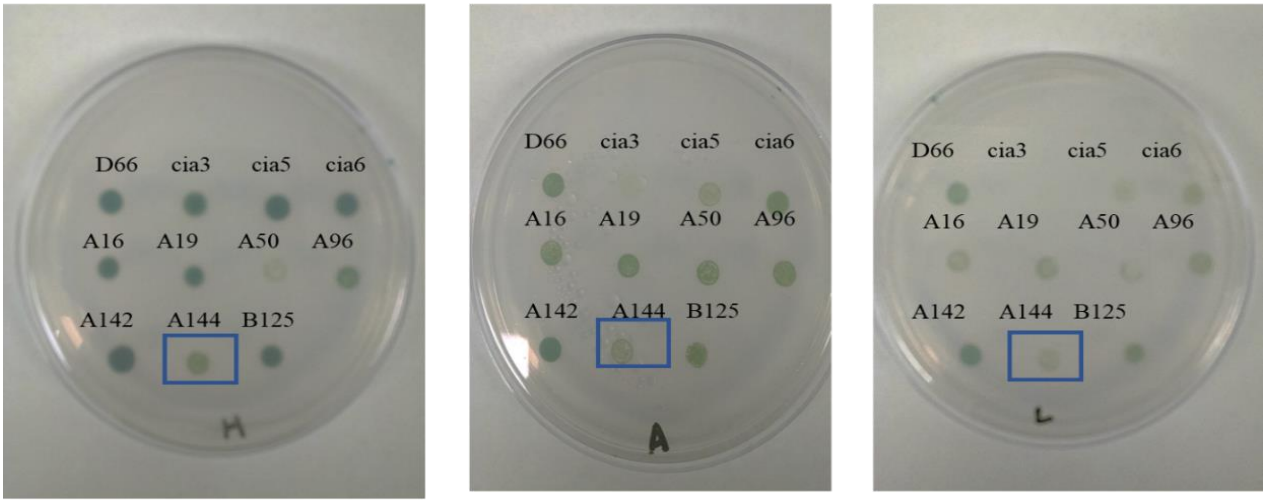


Fig.5.3 Growth of mutants and D66 in different levels of CO_2. This figure shows the growth of some selected mutants along with the wild type and known CCM mutants in low (100 ppm), ambient (400 ppm) and high CO_2 (35, 000 ppm) , A144 has been highlighted in blue for clarity. D66 is the wild type, *cia5* is the mutant for CIA5 transcription factor, *cia3* is the carbonic anhydrase mutant (CAH3) in the thylakoid and *cia6* is a disrupted pyrenoid mutant.

A144 has an insertion in the last exon of the gene

Adapter PCR revealed that A144 has an insert in the gene Cre14.g615950 which contains elongation factor like domains and soluble domains of ATP binding cassette (ABC transporter). The position of the insert was confirmed by making primers flanking the region of the insert, specific to the gene as well as using primers outward from the cassette to confirm both ends of the insert. Both ends of the insert (5' and 3') showed a clean insertion in Cre14.g615950. The gene specific primers amplified a 3kb band when mutant DNA was used as the template which is consistent with the size of the cassette used. Fig. 5.4 shows the image for this experiment.

A144 is a knock out for Cre14.g615950

Both D66 and A144 were grown on TAP for 48 hours followed by overnight growth on high CO_2 as well as low CO_2. RNA was extracted from both sets of cultures and cDNA was made from both high and low CO_2 cells. Primers flanking the insert in the CDS of the gene were used to amplify a 0.4kb region in Cre14.g615950. As shown in fig. 5.5, D66 cDNA does not show any change in expression when the high CO_2 and low CO_2 cells are compared but A144 shows a clear knock out of the gene Cre14.g615950. This figure shows that Cre14.g615950 probably does not play a role in the CCM because it is not induced by lack of CO_2.

Cre14.g615950 is an elongation factor 3 protein

Cre14.g615950 is an elongation factor 3 (EF3) protein which is homologous to fungal EF3 proteins. In Saccharomyces, EF3 mutants are temperature sensitive and therefore fail to survive in higher tempeartures of around 37 °C. EF3 typically has five HEAT repeats (Huntingtin, Elongation factor 3, Protein phosphatase 2A and Yeast kinase TOR1). This is accompanied by soluble domains of ABC transporter proteins. The ABC domains do not have the membrane binding domains in EF3 but only the Walker A and B motifs. There is also a chromodomain or a chromatin organization modifier domain that binds to histones and nucleic acids. The sac7d domain in the chromodomain is resposinble for ATPase activity and 5s tRNA binding. EF3 in Chlamydomonas has 41% identity to EF3 in Saccharomyces. The figure details are summarised in fig. 5.6.

A144 has lower growth and chlorophyll compared to D66

D66 and A144 were grown on both TAP (media with acetate) and MIN (media without a carbon source) for six days. All four cultures were started at a dilution of 0.01 at OD_{730} and the measurements for chlorophyll and OD were taken every 24 hours. A144 grew slower and had less chlorophyll than D66 in both TAP and MIN media, as shown in fig.5.7. This figure shows that A144 is needed for the growth of Chlamydomonas irrespective of a carbon source.

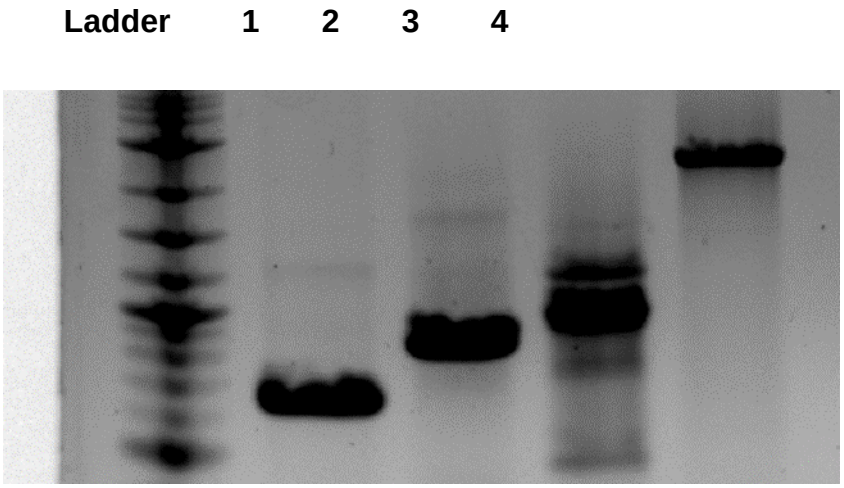


Figure 5.4. The confirmation of the insert in A144. Lane 1 is D66 genomic DNA with primers specific to EF3 gene. Lane 2 is A144 genomic DNA with forward primer specific to EF3 and reverse primer specific to the insert. Lane 3 is A144 genomic DNA with forward primer specific to insert and reverse primer specific to EF3. Lane 4 is A144 genomic DNA with primers specific to EF3 gene.

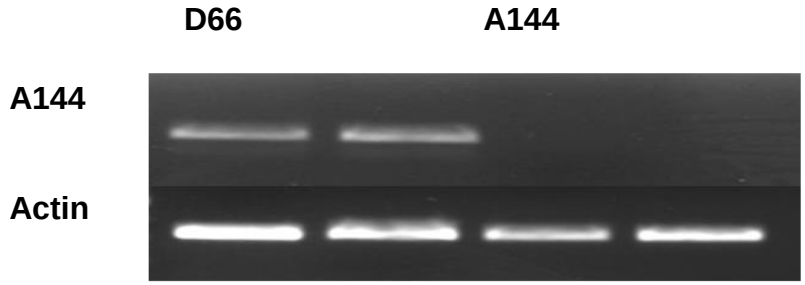


Figure 5.5. Semi quantitative RT-PCR for D66 vs. mutant A144. Accumulation of Cre14.g615950 is shown in both high and low CO_2 grown cells in wild type (D66) and mutant A144. Actin is used as a loading control.

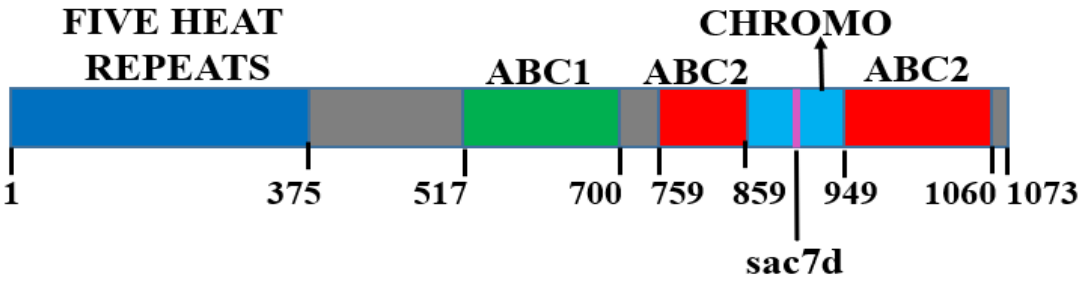


Figure 5.6. The schematic diagram of the domains present in the EF3 gene. The five heat domains are indicated in blue, the two ABC domains are indicated in green and red, the sac7d is indicated in light blue. The black arrow indicates the position of the chromo domain.

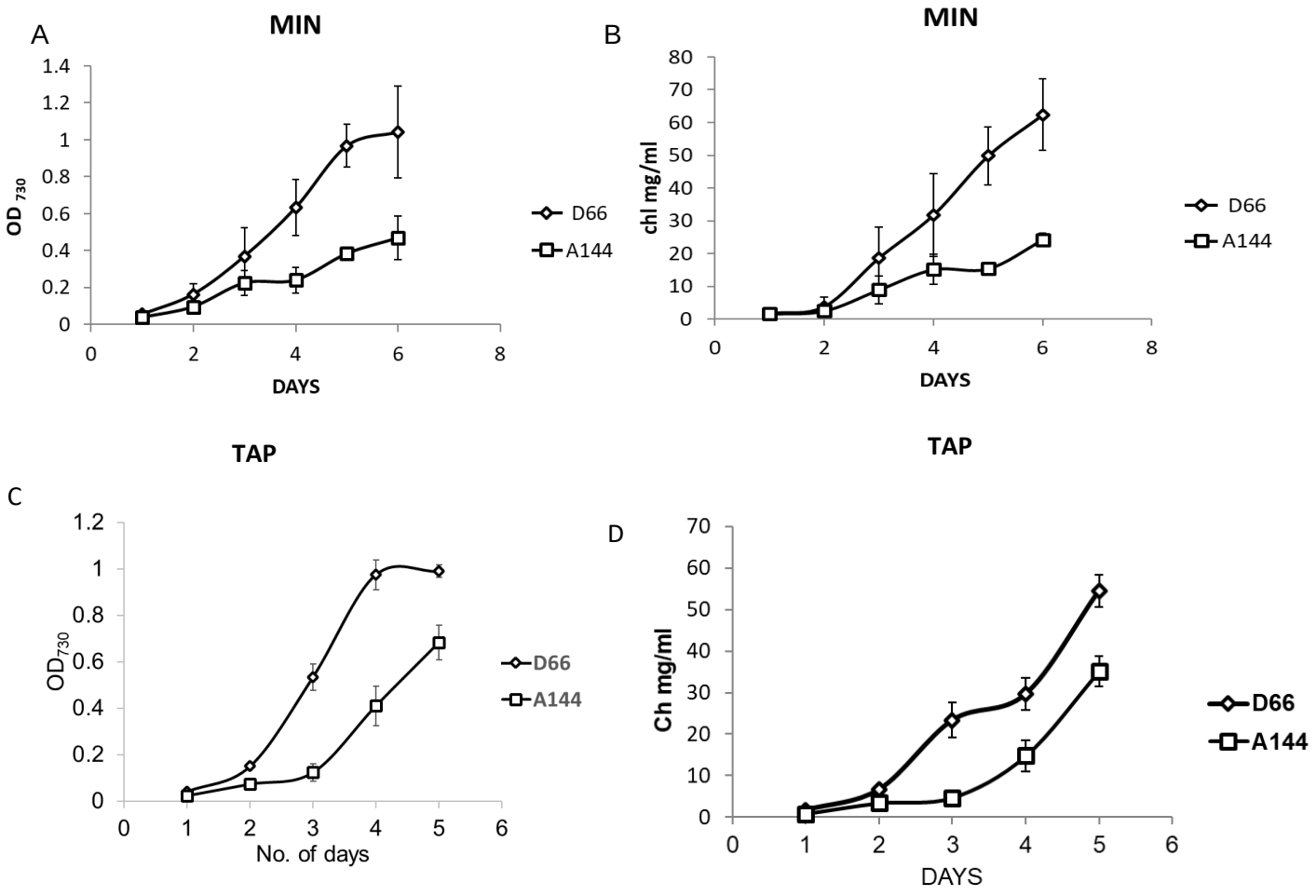


Figure 5.7. Growth difference between D66 and A144 when OD and chlorophyll is measured every 24 hours for 6 days when grown in minimal media A-B) and acetate media C-D). Both cultures were started at OD 0.01 on day 0 in the respective media in ambient levels of CO_2 in 200 μE.

A144 has lower rate of dark respiration than D66

The rate of respiration of both A144 and D66 were calculated in light grown cells and compared to cells that have been dark adapted for 3 days. It was found that while there is no difference in the rate of respiration between A144 and D66 when grown in light, dark adapted cells had a significant difference in rate of respiration. Dark adapted

A144 had a lower rate of respiration than D66. The rate of respiration continued to drop as the period of darkness increased from 3 days to 7 days. Thus Cre14.g615950 plays a role in maintaining the rate of respiration in the dark. The data is shown in fig. 5.8.

Cre14.g615950 is upregulated in transition of light to dark

In order to check if Cre14.g615950 plays a role in transition from light to dark , dark adapted cultures, A144 and D66, were grown in light. A144 stopped growing completely when moved from dark to light unlike D66 which had normal growth and adapted to light well, as shown in fig 5.9A. In order to check if the transcript levels changed for Cre14.g615950 in the dark, RNA was extracted from D66 in light, light to dark transition cells and dark adapted cells to see if there is a change in expression in the transcript of Cre14.g615950. We found that Cre14.g615950 has low expression in light grown cells , high expression in transition phase of light to dark and no expression in dark phase as shown in fig. 5.9B which suggests that Cre14.g615950 plays a role in supporting light to dark transition in cells.

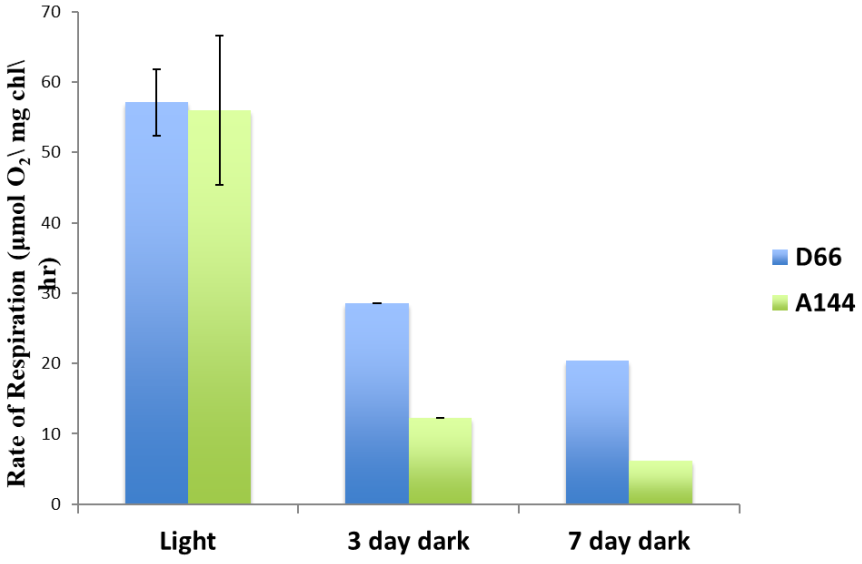


Figure 5.8. This figure shows the difference between respiration rates of D66 and A144. Light respiration had negligible difference between the WT and mutant but dark respiration rates dropped in the mutant after the mutant was dark acclimated for longer periods of time.

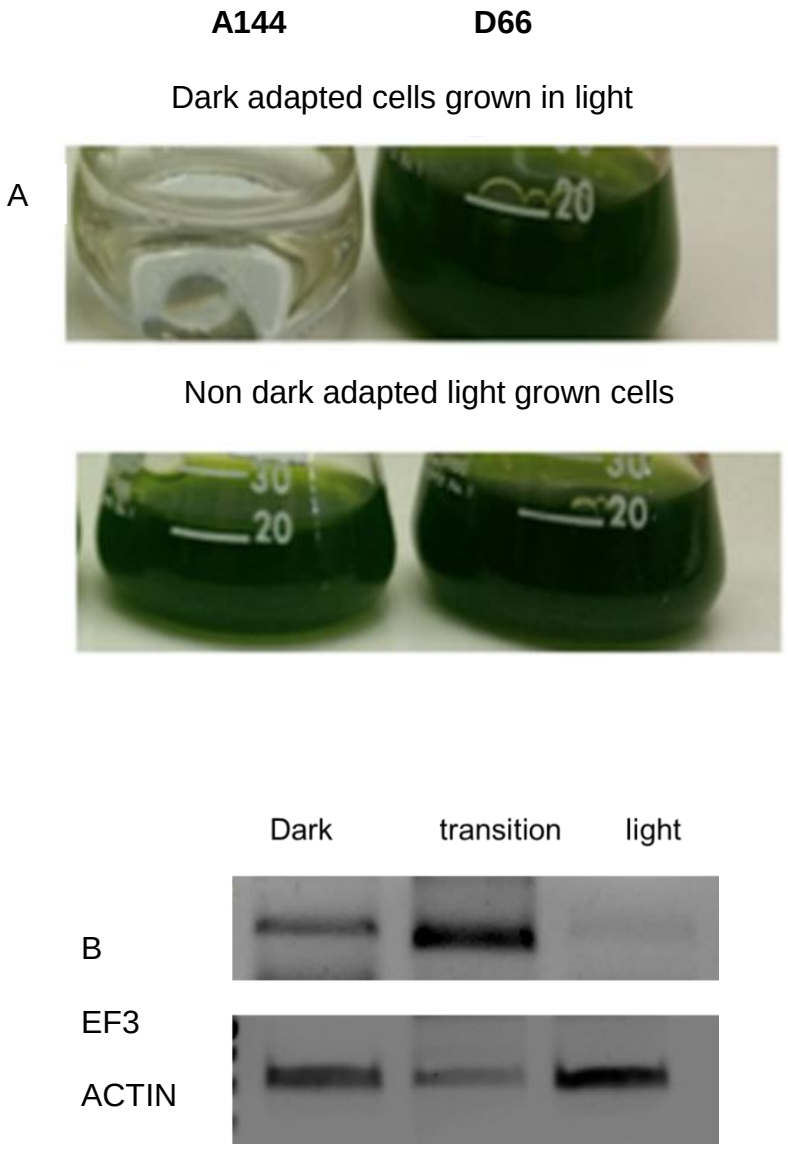


Figure 5.9. A) A144 and D66 are grown in light after dark acclimation. A144 dies but D66 has no such growth defect. Normal light grown cells are used as a control. B) RT-PCR is used to check if the expression of the gene increases when moved from dark to light. Actin is used as a loading control.

Discussion

A large-scale mutant screen was carried out to find novel genes in the CCM of Chlamydomonas. A paromomycin resistant cassette was used, driven by a dual Rubisco small subunit and heat shock protein promoter. Nearly 30,000 mutants were generated from the above transformations from which mutants with a growth defect

were selected. The position of the cassette was determined by our modification of the adapter PCR. In this method we ligated a specific “adapter” to the insert and picked out the flanking gene with adapter specific primers. One such sick mutant had an insertion in elongation factor 3 gene.

Eukaryotic Elongation factor 3 (eEF3) is a member of ATP binding cassette family most of which are involved in transporting substrates across membranes (Hollenstein et al., 2007). eEF3 have soluble domains of ABC transporters which do not have any transportation activity since the membrane binding domains are missing. The internal ATPase activity is mainly stimulated by the ribosome such that mutation of the conserved residues in the ABC domains causes loss of function of the protein. eEF3 also releases, in an ATP dependent manner, deacylated tRNA from ribosomal E site. eEF3 was first discovered in *S. cerevisiae* and has thus far been considered unique to fungi. However, our characterization of the EF3 in Chlamydomonas points to the fact that EF3 is a vital component of Chlamydomonas.

A144, the mutant for EF3, grows slower than the wild type, D66 on minimal media plates. Aside from having lower chlorophyll content, the mutant also grew slower than the wild type in both acetate free and acetate replete liquid media over a period of six days.

In Saccharomyces, a block mutation in the sac7d in eEF3 in yeast prevents the survival of the mutant in higher temperature compared to the wild type. Deletion of eEF3 in yeast is lethal and point mutations in the highly conserved ATP residues make the protein non-functional, compromising ribosome dependent ATP activity (Sasikumar and Kinzy, 2014).

EF3 in Chlamydomonas also has a chromodomain with a sac7d domain with 40% homology to the protein in yeast. In yeast an insertion in the sac7d causes reduced growth rate and lower ribosome stimulated ATPase activity of eEF3 (Sasikumar and Kinzy, 2014). Proteins containing the chromodomain fold have diverse interactions with RNA , DNA and histones etc (Andersen et al., 2006).

The mutant A144 has a similar rate of respiration with the wild type in light but in darkness the respiration rate drops further than the wild type. RT-PCR results also show an upregulation in the expression of EF3 in dark to light transition phase and more expression in dark than light. This is reinforced by growth studies which show that A144 dies in light after prolonged exposure in the dark but no such effect is seen in dark adapted WT as seen in fig. 5.9A. These results suggest that EF3 probably plays a role in stress protein synthesis which is disrupted in A144.

The absence of the elongation factor in the mutant prevents normal dark respiration as well as affects growth rate in green alga, irrespective of growth media. However only further experiments can reveal the exact role EF3 in algae, since no previous work has been done on elongation factor 3 in green algae has been reported. An analysis of stress protein turnover in Chlamydomonas might shed some light on the exact function of EF3. The data suggests that there might be a lack of normal stress protein synthesis in the EF3 disrupted A144 which makes adaptation to dark stressful for the mutant.

CHAPTER 6
CONCLUSIONS

Ci uptake is a vital function of the CCM as it accumulates inorganic carbon several fold higher than the medium. Bicarbonate needs an active transport system for its uptake since unlike CO_2, it does not readily diffuse across membranes. HLA3, LCI1 on the plasma membrane and NAR1.2 on the chloroplast envelope are previously identified bicarbonate transporters that are characterized by upregulation in limiting CO_2 conditions and are generally controlled by CIA5. However, the picture of the CCM in Chlamydomonas is far from complete. For a decade a thylakoid transporter has been hypothesized (Ynalvez et al., 2008) but none had been identified. In addition, the available data on LCIA mutants indicates that there may be more than one chloroplast envelope Ci transporters apart from LCIA. Also, not a lot is known about the signaling and control of the CCM in Chlamydomonas. This thesis identifies several transporters that could putatively play a role in the CCM and characterizes three thylakoid membrane proteins that play a role in the CCM of Chlamydomonas.

There are a number of datasets available in the form of proteomics and transcriptomics which can be used to compile are lists of genes that could be components of the CCM. With this in mind the study in Chapter 3 presents several interesting candidate genes that encode proteins that might play a role in the CCM. This chapter describes a bioinformatics search which aimed to identify putative Ci transporters. The entire proteome of Chlamydomonas was analyzed using predictive software to select for only membrane proteins. These proteins were then categorized based on their domains into several protein families or clans. Only proteins belonging to transporter families were selected for further analysis. Predictive software analysis

followed by cross referencing with RNAseq data condensed the list of proteins to 42 candidates which included the known transporters HLA3, LCIA and LCI1. RT-PCR proved that 9 of the 42 candidates are upregulated in low CO_2 as well as controlled by CIA5. Three such proteins had bestrophin like domains (BST1-3) and were 80% identical to each other with respect to their coding sequence. BST1-3 are described in detail in chapter 4 and characterized further. Another gene of importance is CPLD54 or Gene1, has already been localized to the thylakoid membrane by (Mackinder et al., 2017) and found to be interacting with BST1-3 and LCIB/C. Thus , it will be interesting to knock these genes down along with known transporters or other CCM genes to reveal their function in the Chlamydomonas CCM.

Chapter 4 describes the study of three genes (BST1-3) with bestrophin like domains localized to the thylakoid of Chlamydomonas which are upregulated in low CO_2 as well as controlled by the transcription factor, CIA5. An RNAi approach was undertaken to knock down all three genes as they are very similar. The knockdown mutant with low expression of BST1-3, grows poorly in limiting CO_2 conditions compared to the wild type cells. The mutant also exhibits a poor affinity for inorganic carbon as well as low levels of Ci uptake. A knockout mutant for one of the genes, BST3, was obtained from the CLiP mutant library in Minnesota. *bst3* has no growth defect in low CO_2 conditions and does not show any effect on Ci affinity. This is consistent with NAR1.2 and HLA3 single knockout mutants which only show a difference in Ci affinity when both the genes are knocked out. In a recent interactome study *BST1-3* is shown to be interacting with LCIB/C (Mackinder et al., 2017). Both

these proteins are also shown to interact with each other and another Bestrophin like protein (Cre16.g66400 BST2) (Mackinder et al., 2017).

Bestrophins are generally chloride transporters in Arabidopsis but have also been shown to transport to bicarbonate anions in humans (Qu and Hartzell, 2008). Since our data localizes BST1-3 to the thylakoid membrane, it strongly suggests that these bestrophin-like proteins are the elusive bicarbonate transporter of Chlamydomonas. We were thus able to come up with a new model for the CCM where the new thylakoid Ci transporters BST1-3 were bringing in HCO_3^- from the chloroplast stroma into the thylakoid where CAH3 can dehydrate it to CO_2 (fig.4.12). The CO_2 which leaks out is converted to HCO_3^- by LCIB/C in the stroma and is brought back into the thylakoid by BST1-3. This model hypothesizes that LCIB participates in an anti-leakage recycling mechanism (fig.4.12) as has already been suggested by (Wang and Spalding, 2006; Yamano et al., 2010) with the help of the proton gradient across the thylakoid pump. Thus this thesis has characterized three thylakoid proteins using an RNAi approach to suggest that BST1-3 could be the thylakoid bicarbonate transporter of Chlamydomonas.

Generation and identification of new CCM mutants has also been an integral part of my dissertation work. A large mutagenesis screen was carried out before single mutants (the CLiP library) were readily available, to generate mutants for novel CCM components. This has been summarized in Chapter 5. Our screen generated nearly 22,000 mutants which were screened for a CCM phenotype and the insert location was identified using our modified version of the adapter PCR. Sick on low CO_2 mutants were of primary importance and so the position if the insert was important for us. The adapter

PCR method involved ligating an adapter to restriction digested DNA from the mutants and then carrying out nested PCR with primers specific for the cassette and adapter. PCR products were then sequenced to identify the insert location.

One such mutant with a noticeable growth defect was A144, a knockout for a gene (EF3) with strong homology to elongation factor 3 (eEF3) in fungus. The mutant is sicker than wild type in ambient CO_2 and struggles to survive in light after prolonged dark exposure. A144 also has a low rate of respiration after being exposed to dark for a brief period of time, 3-7 days. Our data also showed that EF3 in Chlamydomonas has higher transcript levels in dark to light transition phase. It was interesting to find a 41% identity in the algal EF3 when compared to the well characterized yeast eEF3 with a conserved sac7d domain. In yeast, a six-block substitution mutant in the sac7d of the eEF3 causes a temperature intolerant mutant. Thus, EF3 in green alga probably synthesizes proteins which plays a role in stress response when cells are moved from dark to light and maintain rates of respiration in the dark. Our insertional mutant sheds light on the role of EF3 in green alga which has not been characterized before. The large scale mutagenesis screen as well as optimization of the adapter PCR helped in finding a mutant for a novel gene that, as per our data, plays an important role in synthesizing dark stress response genes.

Thus this thesis attempts to answer important questions in the CCM of Chlamydomonas using several tools ranging from bioinformatics to RNAi. Our lab also modified the adapter PCR method of finding insertions in Chlamydomonas genes which is another useful tool elucidated in this thesis.

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APPENDIX A
LIST OF PRIMERS IN CHAPTER 3 – 5

Primer name	Sequence 5' → 3'
qBST1F	GCTGTGTGGCATTGAGGAGA
qBST1R	GGATGAGGCTGATGAGTCCG
qBST2F	ACGGTCTACGACTTCCCTCA
qBST2R	TTGGATCACGTGGGATTGGG
qBST3F	AAGTCAGCAAGGTTCCCTCG
qBST3R	TGAATGAGCCTAGCGGGTTG
B2F	CTAGTGAGAGCGTGTTGCAAGGCATATCTCGCTGAT CGGCACCATGGGGGTGGTGGTGATCAGCGCTATATG TTTTGCAACACGCTCTCG
B2R	CTAGCGAGAGCGTGTTGCAAAACATATAGCGCTGAT CACCACCACCCCCATGGTGCCGATCAGCGAGATATG CCTTGCAACACGCTCTCA
B1F	CTAGTGGGAGCGAGTTGCAAGGCATATCTCGCTGAT CGGCACCATGGGGGTGGTGGTGATCAGCGCTATATG TTTTGCAACTCGCTCCG
B1R	CTAGCGGGAGCGAGTTGCAAAACATATAGCGCTGAT CACCACCACCCCCATGGTGCCGATCAGCGAGATATG CCTTGCAACTCGCTCCCA
BST3F	TGCCCCTTCTCAGCACGT
BST3R	ACTGCCTCACACTCCCCT
BST1 RT-F	GACACCAAGACCATCCTGGC
BST1 RT-R	AACAGAACTGCAGAGGTCCCG
BST2 RT-F	CGGTGCCCATGAGCTCC

BST2 RT-R	GCCACTAACCGGCCCAA
BST3 RT-F	AATCCCGTCCATGTCGCT
BST3 RT-R	CGGCTTGTGAGGACCTCG
BST1F	GCTACTCACAACAAGCCCAGTTATGCAGATGCAAGC AAACCGTTCGTC
BST1R	GAGCCACCCAGATCTCCGTTCTTGCGCTCCCCACCC ATGG
BST2F	GCTACTCACAACAAGCCCAGTTATGGCCACTGGTCA GACC
BST2R	GAGCCACCCAGATCTCCGTTTCTCCTTGTCTCCGCAC
BST3F	GCTACTCACAACAAGCCCAGTTATGCAAGTCAGCAA GGTTCCCTCG
BST3R	GAGCCACCCAGATCTCCGTTCCGGGGCGAGATGCG CAC
GENE1 F	CAAGCTACCGCACACAGG
GENE1 R	CATCTCCACAGCCGCTCA
GENE3 F	CGGGTTCTGGGAGCTCTATC
GENE3 R	CACCATTCGGGAGGTGGTC
GENE8 F	TGGCTGTTCATCTGGCTCAA
GENE8 R	ACGGCAACAAACTCCACTTG
GENE13 F	GTTGCTTTCTTGTGCTGCGA
GENE13 R	CTAGGCACATCCTCGTCTCG
GENE17 F	GCGCAGTAAGCACTAAGCAC
GENE17 R	GTTGCTTTCTTGTGCTGCGA
GENE21 F	CGGTGAAGGGGACAGGTAAG
GENE21 R	ACAGGTCGCTGATCAAAGCA
A144 CONFIRMATION F	CACACCCACCCACCAACG
A144 CONFIRMATION R	CCGTCCATCACACGCCCC
A144 RT F	ACGACAAGATCAACGCCGA

A144 RT R	CTCCGCCTTCTCGTCCTTCTC

VITA

Ananya Mukherjee attended West Bengal University of Technology for her Bachelor of Technology degree in biotechnology. Ananya graduated in August 2012 with her bachelor's degree. She joined Dr. James Moroney's lab in the fall of 2012. Her project was to identify and characterize novel Ci transporters especially the elusive thylakoid bicarbonate transporter in Chlamydomonas. Ananya is a candidate to receive her PhD in Biological Sciences in spring of 2019 and will be joining a postdoctoral position in University of Nebraska-Lincoln in summer.